

# Microbiology of Environmental Engineering Systems

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**Abstract** Type of energy generation is the major feature in physiological classification of prokaryotes. Chemotrophs can be separated within four groups by the type of electron acceptor: (a) anaerobic fermenting prokaryotes, producing biologically available energy by intramolecular oxidation-reduction; (b) anaerobically respiring prokaryotes, using other than oxygen electron acceptors; (c) microaerophilic bacteria, producing energy by aerobic respiration at low concentration of oxygen; (d) obligate aerobes, producing biologically available energy with oxygen as electron acceptor. There are also intermediary subgroups, which are using different types of energy production, depending on conditions. Phototrophs also can be classified into related physiological groups by the type of electron donor: (a) electron donors are products of anaerobic fermentation (organic acids, alcohols, and H<sub>2</sub>); (b) electron donors are products of anaerobic respiration (H<sub>2</sub>S, Fe<sup>2+</sup>); (c) electron donors are products of microaerophilic respiration (S); (d) electron donors are products of aerobic respiration (H<sub>2</sub>O). To overcome contradiction between the physiological groups and rRNA gene sequencing-based phylogenetic groups, the periodic table of prokaryotes comprising and explaining the existence of all physiological groups of prokaryotes was proposed. The main feature of the periodic table of prokaryotes is three parallel phylogenetic lines: (a) prokaryotes with Gram-negative type cell wall, habiting mainly in aquatic systems with stable osmotic pressure; (b) prokaryotes with Gram-positive type cell wall, habiting mainly in terrestrial systems with varied osmotic pressure; (c) Archaea that lack conventional peptidoglycan and habiting mainly

in extreme environments. There are four periods in the periodic table of prokaryotes: anaerobic fermentation, anaerobic respiration, microaerophilic respiration, and aerobic respiration. Three phylogenetic lines and four periods create 12 groups comprising all chemotrophic and phototrophic prokaryotes. Existence of Gram-positive phototrophic bacteria using products of anaerobic, microaerophilic, and aerobic respiration as electron donors was predicted using this periodic table of prokaryotes. Evolutionary parallelism in phylogenetic lines of prokaryotes could be hypothetically explained by synchronous evolution of aquatic, terrestrial, and extreme ecosystems and horizontal exchange of genes between these ecosystems. The periodic table of prokaryotes helps to understand microbial physiological diversity of environmental engineering systems and can be used in the design of environmental engineering processes.

**Key Words** Prokaryotes • physiological classification • evolutionary parallelism • environmental engineering systems.

## 1. MICROBIAL GROUPS AND THEIR QUANTIFICATION

Microbiology is a branch of biology devoted to the study of microorganisms (microbes), which include both unicellular and multicellular organisms. These microorganisms are not visible without the aid of a microscope because they are smaller than 70–100  $\mu\text{m}$ . Microbiological sciences, such as industrial microbiology, medical microbiology, veterinary microbiology, agricultural microbiology and environmental microbiology, are specified by their objects of study. Environmental microbiology studies microbes in parts of biosphere such as lithosphere, hydrosphere and atmosphere. The microbiology of environmental engineering systems is a subset of environmental microbiology. The objects of this science are the engineering systems of water, wastewater, solid wastes, soil and gas biotreatment.

The microbiology of environmental engineering systems pursues practical goals such as:

1. Development of biotechnologies for the microbial treatment of water, wastewater, solid wastes, soil and gas.
2. Development of methods to prevent the outbreaks of water-borne, soil-borne, vector-borne, and airborne infectious diseases.
3. Development of methods to monitor and control environmental engineering systems.

However, achievement of such practical goals is not possible without studying the following general problems of environmental microbiology:

1. Classification and identification of microorganisms.
2. Physical, chemical and biological interactions between microorganisms and macroorganisms.
3. Physical and chemical interactions of microorganisms and environment.
4. Biochemical, physiological and cellular adaptations and regulations in microbial systems.

This chapter is intended for environmental engineers as well as environmental engineering students who do not possess an in-depth microbiological background. We will address the basic principles of microbiology of environmental engineering systems, with special attention paid to the interconnections and diversity of microbial groups as well as their functions in

environmental engineering systems. A more in-depth description of these topics is given in specialized chapters of this book.

### 1.1. Groups of Microorganisms

The objects of the microbiology of environmental engineering systems include bacteria (prokaryotes), microscopic fungi, microscopic algae, protozoa and other microscopic objects such as viruses, metazoa and cysts of the helminthes. All living organisms are composed of cells. Prokaryotic cells are relatively simple in structure; they lack a true nucleus covered by the membrane. The most common cell shapes are spherical and rod-shaped. A eukaryotic cell's structure is more complex because it contains organelles that serve as compartments for special metabolic functions.

*Viruses* are particles assembled from the biopolymers, which are capable of multiplying and assembling as new virus particles inside living prokaryotic or eukaryotic cells. Viruses are not traditionally included in biological classifications because they are obligate intracellular parasites of cells, and thus, cannot self-reproduce. Extracellular virus particles are metabolically inert. The typical virus size ranges from 0.02 to 0.2  $\mu\text{m}$ . Viruses contain a single type of nucleic acid, either DNA or RNA. There are known virus-like agents called prions, which are infectious proteins. Viruses are important for environmental engineering because of the following reasons:

1. Pathogenic viruses must be removed, retained or destroyed during water and wastewater treatment.
2. Viruses of bacteria (bacteriophages) can infect and degrade the bacterial cultures.
3. Bacteriophages can be used for the detection of specific microbial pollution of environment.
4. Viruses may be a vector (carrier) of the genes in artificial or natural genetic recombinations.

*Prokaryotes* are microorganisms with prokaryotic type cells. They consist of two phylogenetic groups: Bacteria and Archaea. The typical size of these cells is between 1 and 2  $\mu\text{m}$ , but there have been cells known to be smaller or bigger than this range. Prokaryotes are most active in the degradation of organic matter and are used in wastewater treatment and soil bioremediation. However, there are many bacteria that are harmful to human, animal and plant health, and the removal or killing of these pathogenic bacteria in water, wastewater or solid waste is an important task of environmental engineering.

Energy sources for the growth of prokaryotes include:

1. Chemical substances (chemotrophy) or light (phototrophy).
2. Utilization of organic substances (heterotrophy) or inorganic substances (lithotrophy).

Other physiological properties also vary:

1. Source of carbon may be carbon dioxide (autotrophy) or organic substances (organotrophy).
2. Optimal temperature for growth varies from 0°C to higher than 100°C.
3. Optimal pH for growth varies from two to nine.

*Relation to oxygen* is one of the main features of prokaryotes. Generation of biologically available energy in a conducted cell is due to oxidation–reduction reactions. Oxygen is the most effective acceptor of electrons in energy generation from oxidation of substances, but not all

microorganisms can use it. The following groups of microorganisms differ in their relation to oxygen:

1. Obligate anaerobic prokaryotes, producing energy by fermentation (it is intramolecular oxidation–reduction without an external acceptor of electrons); they die after contact with oxygen because they lack protection against oxygen radicals produced during the contact of cells with oxygen.
2. Tolerant anaerobes produce energy only by fermentation but survive after contact with oxygen due to protective mechanisms against oxygen radicals.
3. Facultative anaerobic bacteria, which are capable to produce energy by fermentation if oxygen is absent or by aerobic respiration if oxygen is present.
4. Microaerophilic bacteria, which prefer low concentration of dissolved oxygen in a medium.
5. Obligate aerobes produce energy by aerobic respiration only.

*Anoxic (anaerobic) respiration* is typical for prokaryotes only and is the oxidation of organic or inorganic substances by electron acceptors other than oxygen. Different electron acceptors are used for energy generation by specific physiological groups of prokaryotes, including:

1. Nitrate ( $\text{NO}_3^-$ ) and nitrite ( $\text{NO}_2^-$ ) are used by denitrifying bacteria (denitrifiers).
2. Sulphate ( $\text{SO}_4^{2-}$ ) is used by sulphate-reducing bacteria.
3. Sulphur (S) is used by sulphur-prokaryotes.
4. Ferric ions ( $\text{Fe}^{3+}$ ) is used by iron-reducing bacteria.
5. Ions of different oxidized metals are used as acceptor of electrons.
6. Carbon dioxide ( $\text{CO}_2$ ) is used by methanogens.

*Fungi* are eukaryotic microorganisms, mostly multicellular, which assimilate organic substances and absorb nutrients through the cell surface. The typical cell size is between 5 and 20  $\mu\text{m}$ . Cells are often combined in the branched filaments called hyphae, which are combined in a web known as mycelium. Fungi are important degraders of polymers and are used in the composting and biodegradation of toxic organic substances. Fungi are used in environmental engineering in composting, soil bioremediation and biodegradation of xenobiotics. Mycelium effectively penetrates solid wastes and soil. There are five major groups of fungi:

1. *Oomycetes* (water molds).
2. *Zygomycetes* (molds).
3. *Ascomycetes* (sac fungi and yeasts) reproduced by spores stored in the sac called ascus or spores called conidia.
4. *Basidiomycetes* (club fungi and mushrooms).
5. *Deuteromycetes* (or *Fungi imperfecti*) have no known sexual stage.

Molds are filamentous fungi (from *Zygomycetes* and *Ascomycetes*) that have widespread occurrence in nature. They have a surface mycelium and aerial hyphae that contain asexual spores (conidia). These spores are airborne allergens in damp or poorly constructed buildings. Yeasts (from *Ascomycetes*) are fungi that grow as single cells, producing daughter cells either by budding (the budding yeasts) or by binary fission (the fission yeasts). Mushrooms are filamentous fungi that form large above-ground fruiting bodies, although the major portion of the biomass consists of hyphae below ground.

Algae are floating eukaryotic microorganisms that assimilate energy from light. The typical size of a cell is 10–20  $\mu\text{m}$ . Algae carry out oxygenic photosynthesis:



Algae live primarily in aquatic habitats and on the soil surface. Algae should not be confused with cyanobacteria, which are prokaryotes. The classification of algae is based on the type of chlorophyll and other pigments, cell wall structure and nature of carbon reserve material:

1. *Chlorophyta* (green algae).
2. *Chrysophyta* (golden-brown algae).
3. *Euglenophyta*, have no cell.
4. *Pyrrophyta* (dinoflagellates).
5. *Rhodophyta* (red algae).
6. *Phaeophyta* (brown algae).

Algae are important for environmental engineering for the following reasons:

1. They remove nutrients from water and are active microorganisms in waste stabilization ponds.
2. Some algae are fast-growing in polluted water and produce toxic compounds; these cause the “red tides” in polluted coastal areas.
3. Selected species of microscopic algae in natural waters are used for the indication of water quality.
4. There may be value-added products, for example, pigments and unsaturated fatty acids from algae grown in wastewater.

*Protozoa* are unicellular organisms that absorb and digest organic food inside a cell. The typical cell size is from 10 to 50  $\mu\text{m}$ . Some protozoa are pathogenic and must be removed from water and wastewater. Four major groups of protozoa are distinguished by their mechanism of motility: amoebas move by means of false feet; flagellates move by means of flagella; ciliates use cilia for locomotion; and some protozoa have no means of locomotion. Examples are given in Table 2.1.

Protozoa are unicellular organisms that obtain nutrients by ingesting other microbes, or by ingesting macromolecules. The cells form cysts under adverse environmental conditions

**Table 2.1**  
**Examples of parasitic protozoa**

Group	Example of parasitic species from this group	Disease caused by this species
<i>Sarcodina</i> (amoeboids)	<i>Entamoeba histolytica</i>	Amebiasis
<i>Mastigophora</i> (flagellates)	<i>Giardia intestinalis</i>	Giardiasis
<i>Ciliophora</i> (ciliates)	<i>Balantidium coli</i>	Balantidiasis
<i>Sporozoa</i> (no means of locomotion)	<i>Plasmodium vivax</i> <i>Cryptosporidium spp.</i> (more than ten species)	Malaria Cryptosporidiosis

and are resistant to desiccation, starvation, high temperature and disinfection. Changes in the protozoan community reflect the operating conditions of aerobic wastewater treatment:

1. Amoebas can be found in high concentrations of organic matter (at high values of biochemical oxygen demand – BOD).
2. Flagellated protozoa and free-swimming ciliates are associated with high bacterial concentrations in activated sludge and medium concentration of BOD values.
3. Protozoa contribute significantly to the reduction of bacteria, including pathogens in activated sludge.
4. Stalked ciliates occur at low bacterial and BOD concentrations in water.

*Helminthes* are parasitic worms that survive in humans and animals. Many of these parasitic worms have microscopic cysts (seeds). The removal or inactivation of these cysts in water, wastewater and solid wastes is a goal of environmental engineering. Due to the high hydrophobicity of the cyst surface, cysts can be accumulated in the landfill leachate, foam of aeration tanks, or float up during the storage or primary treatment of sewage.

## 1.2. Microbiological Methods Used in Environmental Engineering

*Specific microbiological methods* are used to study microorganisms in environmental engineering systems:

1. Isolation, cultivation, identification and quantification of pure cultures.
2. Selection of strains and construction of recombinant microbial strains.
3. Selection and quantification of enrichment cultures.
4. Identification and quantification of microorganisms in environmental samples without cultivation.
5. Extraction, cloning, enrichment and identification of microbial genes and their products in environmental engineering systems.

*Isolation of pure culture* (microbial strain) is usually performed by spreading a diluted microbial suspension on a Petri dish with a semi-solid medium to produce 10–50 colonies on the dish after several days of cultivation. Cells of one colony are picked up for the next round of cultivation on a semi-solid or liquid medium. However, the following methods can also be effectively used for the isolation of pure microbial culture:

1. Mechanical separation of cells by micromanipulator.
2. Sorting of cells or microbeads with immobilized cell, using flow cytometer.
3. Magnetic or immunomagnetic separation.
4. Cell chromatography.

A microbial population that originates from one colony is called a microbial strain. A microbial population that originates from one cell is called a microbial clone.

*Selection* is the screening of microbial variants with specific desirable characteristics within the population of one strain. These variants may include:

1. Faster or more efficient growth (positive selection).
2. Faster or more efficient biochemical function (positive selection).
3. Slower or less efficient biochemical function (negative selection).
4. Better survival under harmful conditions (positive selection).
5. Weaker resistance to some factors of the environment (negative selection).

The differences between variants are caused by natural spontaneous mutations, i.e., changes in the DNA sequences of genes. Mutagenic chemicals, ultraviolet rays and ionizing radiation are used to increase the rate of mutagenesis and to increase probability of desirable variant formation. The screening of the desirable variant can be replaced by the creation of selection pressure, i.e., conditions favorable for growth, survival or development of desirable variant. Therefore, this variant will be accumulated in a microbial population and can be detected during the screening.

*The cultivation* of microorganisms is performed under suitable conditions, usually at optimal temperature, pH, osmotic pressure and concentration of gases (oxygen, carbon dioxide, hydrogen), on a semi-solid or liquid medium, containing all necessary substances for the growth of the strain. The elemental composition of biomass can be shown approximately by the formula  $\text{CH}_{1.8}\text{O}_{0.5}\text{N}_{0.2}$ , but half of the known elements are used in the synthesis of microbial biomass and must be present in the medium. Suitable conditions and essential substances for the growth of some strains are not known yet and the cultivation of these microorganisms has not been successful to date. Additionally, some microorganisms are living in strong symbiotic or parasitic relationships with other microorganisms or macroorganisms that cannot be cultivated separately from these organisms. Therefore, not all microorganisms can be isolated and cultivated.

*The identification* of microorganisms involves the determination of relationship of a studied strain (taxon) with some known group, which is then accepted and approved by an international scientific committee. Measurement or qualitative evaluation of the relationship between compared microbial groups is performed by the methods of phenotypic classification (conventional taxonomy) and genotypic classification (phylogenetic taxonomy).

*Phenotypic classification* (conventional taxonomy) is based on the phenotypic characteristics, i.e., visible or measured characteristics determined by an organism's interaction with the environment:

1. Cytological characteristics such as size, shape, cell structure, typical cell aggregates, membrane structures, intracellular structures and cell organelles. One of most important cytological characteristics of prokaryotes is the Gram-positive or Gram-negative type of cell wall. A Gram-positive cell wall is a thick and rigid 3-D layer of polymer. A Gram-negative cell wall is a thin and more elastic layer of polymer, which is covered by an outer membrane and a lipopolysaccharide layer.
2. Physiological characteristics such as type of energy production, relation to oxygen, pH, temperature, chemical content of cell wall and membranes, production of specific metabolites and enzyme profile.
3. Ecological characteristics such as habitats, niches, colonial structures and interrelationships with other organisms.

*Genotypic classification* (phylogenetic taxonomy) is based on the analyses of genetic characteristics of the organisms, which are stored in the sequences of DNA. Genotypic characteristics include:

1. G + C content in DNA.
2. Sequences of genes (the sequences of DNA, which store information on the biopolymers of homologous, similar function in different species are compared).
3. Sequences of homologous (similar) proteins.
4. Level of hybridization between the sequences of DNA and RNA of compared strains.

*Collections of strains and clones.* The properties of strains and clones are the primary data used for classification. Strains are stored in microbial collections in the form of suspension, colonies on solid medium, or in dry or frozen state. There are many specialized and national collections of microorganisms. The purpose of such collections is to acquire, authenticate, preserve, develop and distribute biological materials, information, technology, intellectual property and standards for the advancement, validation and application of scientific knowledge to private industry, government and academic organizations. For example, large culture collections include the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and American Type Culture Collection (ATCC). A strain is identified by its assigned number in a microbial collection and the name of the species. For example, *Bacillus subtilis* ATCC6633 refers to a strain of species *Bacillus subtilis* stored under number 6333 in the ATCC.

A *species* is a primary unit of phenotypic classification. It is defined by the phenotypic and genotypic characteristics of a collection of similar strains. The name of any species is given and read in *Latin* and includes the name of the genus (first) and the name of the species (second): *Saccharomyces cerevisiae*, *Bacillus subtilis*.

*Higher levels of phenotypic classification* are genus (collection of similar species), family (collection of similar genera), order (collection of similar families) and kingdom (collection of similar orders). Prokaryotic groups of conventional taxonomy are described in Bergey's Manual of Systematic Bacteriology (1). The manual contains phenotypic characteristics, which are used to classify prokaryotes by conventional taxonomy. Groups of viruses, fungi, protozoa and algae are described in specific manuals approved by related international scientific associations. Prokaryotic groups of phylogenetic taxonomy can be defined through the comparison of the sequences of ribosomal RNA (rRNA), especially 16S rRNA. It is thought that the number of differences in the sequence reflects the evolutionary distance of the origin of compared sequences from a common ancestor sequence.

*Selection of enrichment culture* refers to the selection of the population with one dominated strain or one dominated microbial community, which is accumulated in the system of cultivation because of the preferred conditions (selection pressure) for this strain or community. Enrichment cultivation is often used in environmental engineering to select microorganism(s) capable of particular metabolic transformations. Selective conditions (selection pressure) for the production of enrichment culture are as follows:

1. Source of energy.
2. Source of carbon.
3. Source of nitrogen and phosphorus.
4. Temperature.
5. pH.
6. Concentration of heavy metals.
7. Presence of specific antibiotic in a medium.
8. Concentration of dissolved oxygen.
9. Osmotic pressure of a medium.
10. Spectrum and intensity of light, etc.

*Quantification of microbial biomass*, i.e., determination of cell number (enumeration) or quantity of cell biomass, can be performed by the following methods:



1. Microscopic or flow cytometric enumeration of cells.
2. Physical measurement of microbial cells and biomass concentration.
3. Chemical measurement of microbial cells and biomass concentration.
4. Biological methods of cell and viruses enumeration.
5. Physiological measurement of biomass.
6. Molecular-biological methods of cells and viruses enumeration.

Factors that affect the choice of the method include:

1. Cost and length of time required for analysis.
2. Sensitivity and specificity of the method.
3. Availability of the equipment.
4. Characteristics of the interest.

*Microscopic enumeration* of cells and virus particles is performed using light microscopes (bright field, phase contrast and fluorescence microscopy), confocal laser scanning microscopes (CLSM), transmitted electron microscopes (TEM), scanning electron microscopes (SEM) and other kinds of microscopes that are able to visualize a number of microbial cells or virus particles on a defined area. The particular cell structure may be specifically stained, cell or virus surface may be labeled by immunochemical methods and DNA or RNA of cells can be hybridized with oligonucleotide probes labelled by fluorescence, radioactive or other labels. This specific staining can ensure cell enumeration altogether, with cell identification and measurement of cell physiological state.

*Flow cytometry enumeration* is used to quantify cells by staining specific cells with fluorescent-labelled antibodies, oligonucleotide probes and specific fluorochromes, which are excited by lasers in the flow of a small diameter (2). The fluorescence of individual cells is then measured by photomultipliers and the signals are collected and treated by a computer. In addition to cell number, three to six other parameters of thousands of individual microbial cells or virus particles can be analyzed in seconds.

*Physical methods for microbial biomass measurement* are based on the determination of weight, optical density, turbidity, fluorescence or radioactivity of microbial suspensions and solid matter. A convenient method of suspended biomass estimation is turbidity measurement. The share of scattered light is proportional to the cell concentration in the sample. Autofluorescence of microbial cell components (chlorophyll of algae, bacteriochlorophyll and carotenoids of cyanobacteria,  $F_{420}$  of methanogens) or fluorescence of stained cells also can be used for the measurement of biomass. Fluorescence spectrometry can be used to quantify microorganisms in environmental engineering systems, using determination of the binding of specific oligonucleotide probes (3).

*Chemical methods for microbial biomass measurement* include the analysis of protein, DNA or the components of cell wall, ATP, photopigments, cytochromes, coenzymes  $NADH_2$  or  $F_{420}$ . ATP measurement is a sensitive indicator of a small quantity of viable microorganisms. The chemical changes in the medium caused by the microbial growth can be monitored using electrochemical sensors and fiber optic sensors.

*Physiological methods of microbial biomass measurement* are based on the measurements of physiological activity of the cells, for example, respiration rate, biochemical transformation rate and ATP concentration.

*Biological methods of cell enumeration* are as follows:

1. Plate count, i.e., cultivation on a semi-solid medium and enumeration of colony-forming units (CFU). It is assumed that one cell produces one colony, but this assumption is often not right. There may be  $10^3$ – $10^{12}$  cells in 1 mL of the sample. Therefore, it should be diluted in a sterile medium before being spread onto a Petri dish to produce not more than 100–300 colonies per plate.
2. Most probable number count, i.e., identification of the maximum dilution at which the growth or microbial activity can be easily detected by the colour change, precipitation or formation of gas bubbles. For example, if the maximum dilution to detect microbial activity in 1 mL of specific medium is  $5 \times 10^{-4}$ , the most probable number of cells in the sample is  $2 \times 10^3$  cells/mL. It is assumed that multiplication of one cell in the tube with maximum dilution can produce the detectable result (color change, gas bubbles), but this assumption is often incorrect.

If the studied cells and viral particles have a low cell concentration, they must be concentrated using the following methods:

1. Filtration of the sample through a sterile membrane filter having a pore size  $< 0.45 \mu\text{m}$  to retain bacterial cells and  $< 2\text{--}5 \mu\text{m}$  to retain eukaryotic cells.
2. Precipitation or centrifugation of cells or viral particles of the sample.
3. Chromatography of the sample.
4. Adsorption of cells and viral particles in the column with a specific adsorbent.

Viruses are enumerated biologically by spreading a diluted suspension on the surface of a lawn of actively growing cells susceptible to the virus. As a virus particle infects and reproduces, the produced viruses kill surrounding cells, forming a zone of clearing in the cell layer.

*Molecular-biological methods for microbial biomass quantification* are as follows:

1. Immunochemical quantification of microbial biomass due to colour change in the reaction between specific antibody(s) and the cell surface.
2. Molecular-biological quantification of microbial biomass due to color change in the in situ reaction between specific oligonucleotide probe(s) and cell RNAs or DNA (3).
3. Quantitative polymerase chain reaction (PCR) called real-time PCR. It involves the extraction of DNA from the sample and amplification of specific genes with its quantification after every cycle of amplification. This method is especially important for bacterial groups that cannot be cultivated in the laboratory because the medium or growth conditions for them were not yet defined, or which are symbiotic or parasitic species.

Viruses can be enumerated by immunochemical methods or by PCR of specific DNA/RNA.

### ***1.3. Comparison of Physical, Chemical, Physico-chemical and Microbiological Processes***

Environmental engineering problems, i.e., waste or wastewater treatment, soil bioremediation and biopurification of exhaust gases, can be usually solved by physical, chemical, physico-chemical and biological/microbiological technologies. An optimal technology can

**Table 2.2**  
**Advantages and disadvantages of different environmental engineering technologies**

Type of technology	Advantages	Disadvantages
Physical technologies (sedimentation, filtration, volatilization, fixation, evaporation, heat treatment, radiation, etc.)	Required time is from some seconds to some minutes; high predictability of the system	Low specificity and high energy demand
Chemical technologies (oxidation, incineration, reduction, chemical immobilization, chelating, chemical transformation)	Required time is from some seconds to some minutes; high predictability of the system	High expenses for reagents, energy, and equipment; air pollution due to incineration, formation of secondary wastes
Physico-chemical treatment (adsorption, absorption, chromatography)	Required time is from some minutes to some hours	High expenses for adsorbents; formation of secondary waste
Microbiological technologies (biooxidation, biotransformation, biodegradation)	Low volume or absence of secondary hazardous wastes; process can be initiated by natural microorganisms or small quantity of added microbial biomass; high process specificity; wide spectrum of degradable substances and diverse methods of biodegradation	High expenses for aeration, nutrients, and maintenance of optimal conditions; required time is from some hours to days; unexpected or negative effects of microorganisms- destructors; low predictability of the system because of complexity and high sensitivity of biological systems

be selected, based on economical or environmental criteria. Some general advantages and disadvantages of different environmental engineering technologies are shown in Table 2.2.

## 2. MICROBIAL ECOSYSTEMS

### 2.1. Structure of Ecosystems

An *ecosystem* comprises biotic (biological) and abiotic (physical, chemical) components, interacting with each other and isolated from the environment by a boundary.

The *hierarchy of life units* in microbial ecosystems can be represented in order of increasing spatial and biological complexity of ecosystems and the sequence of their combination:

1. Suspended cells (unicellular organisms) of one species.
2. Suspended cells (unicellular organisms) of microbial community.
3. Aggregated cells and multicellular microorganisms.
4. Ecosystems of located biotop.
5. Ecosystems of whole biosphere.

The *boundary* between an ecosystem and its surrounding environment is a steep gradient of physical and/or chemical properties. The physical boundary is formed by an interphase between solid and liquid phases, solid and gas phases, liquid and gas phases. For example, the microbial ecosystem of an aerobic tank for wastewater treatment is separated from the environment by the reactor walls and air–water interphase. The steep gradient of chemical substances, for example, oxygen, ferrous, hydrogen sulphide, etc., forms a chemical barrier. Such barriers separate, for example, aerobic and anaerobic ecosystems in a lake. The steep gradient of conditions can also be created by cell aggregation in flocs, granules or biofilms. The main function of the boundary is to maintain integrity of an ecosystem by controlled isolation from the environment, and to protect an ecosystem from the destructive effects of the environment.

*The boundaries of unicellular organism* are as follows:

1. The cell membrane (cytoplasmic membrane) performs selective and controlled exchange of molecules between cell and environment. It is the most sensitive boundary because even a small break in the cell membrane will destroy the isolating and energy-generating properties of a cell membrane. Surface-active substances, organic solvents, oxidants and high temperature destroy the integrity of a cell membrane.
2. The cell wall protects a cell from changes in osmotic pressure and mechanical impulses. Bacteria with a thick cell wall are stained as Gram-positive cells.
3. Bacteria that are stained as Gram-negative cells have a thin cell wall covered by an outer membrane. Lipopolysaccharides of outer membrane of Gram-negative bacteria are very specific. These molecules interact with the human body's immune system and are often toxic or allergenic for humans. Gram staining is just one, and not always reliable method to differentiate bacteria with Gram-positive and Gram-negative types of cell walls.
4. Some prokaryotes, for example, mycoplasmas, have no cell wall.
5. Fungi and algae often have cell walls containing polysaccharides such as cellulose or chitin. Some algae have inorganic compounds such as calcium carbonate or silica in their rigid walls. Animal cells often have no cell walls.
6. The glycocalyx (capsule) is an extracellular polysaccharide, covering microbial cells of some species. Its functions include attachment of the cells to the surface; aggregation of cells; protection of cells against drying, oxidants, heavy metals and antibiotics.

*A multicellular aggregate* is formed and separated from its surrounding environment due to:

1. Aggregation by hydrophobic force, electrostatic interactions or salt bridges.
2. Loose polysaccharide or inorganic matrix (iron hydroxide, for example), combining the cells altogether by mechanical embedding, chemical bonds, hydrogen bonds, electrostatic forces or hydrophobic interactions.
3. Formation of mycelia, which is a net of branched cell filaments.
4. Polysaccharide matrix with a filamentous frame.
5. Structured matrix with layers parallel to the boundary or subaggregates, which are perpendicular to the boundary (4).
6. Coverage by a common sheath of organic (polysaccharides, proteins) or inorganic origin (iron hydroxide, silica, calcium carbonate).
7. Coverage by a common sheath ("skin" of microbial aggregate) consisting of dead cells.

A microbial aggregate can be considered as a multicellular organism if its parts have different coordinated or synchronized physiological functions, i.e., growth, motility, sexual interactions, assimilation of atmospheric nitrogen, production of extracellular polysaccharides, transport and distribution of nutrients and reduction of oxygen.

*Microbial communities of environmental engineering systems* are usually suspended or adhered to surface cells and microbial aggregates such as fixed biofilms and suspended flocs or granules. The boundaries of these ecosystems are as follows:

1. Side walls of the equipment with a fixed microbial biofilm.
2. Bottom of the equipment with the sediment of microbial aggregates.
3. Gas–liquid interphase with accumulated hydrophobic substances (lipids, hydrocarbons, aromatic aminoacids) and cells or aggregates with high hydrophobicity of their surface or cells and aggregates containing gas vesicles (5).

*Diversity of a microbial ecosystem* refers to the heterogeneity of genotypes (diversity of strains, species, physiological groups), in space (different zones, layers, aggregates and chemical or physical gradients), and in time (temporal changes in diversity of genotypes and spatial structure of ecosystem). Succession refers to the typical sequence of temporal changes in an ecosystem. Stagnation or climax is a state of ecosystem characterized by weak changes caused by poor environment, degeneration or ageing of the system.

There are known numerous mathematical expressions to quantify diversity. For example, Shannon-Weaver index ( $H$ ) is:

$$H = \sum_{i=0}^{i=S} [p_i - \ln(p_i)] \quad (2)$$

where  $p_i$  is the proportion of the  $i$ -th group in the community, and  $S$  is a number of the groups in the community. Evenness index ( $E$ ) is a measure of how similar the abundances of different groups are:

$$E = H / \ln S \quad (3)$$

When there are similar proportions of all groups, then the evenness index is one. The evenness index is larger than one when the abundances are very dissimilar. An example of quantitative characterization of microbial diversity in an anaerobic digester of activated sludge is given below.

*Example: diversity in an anaerobic digester.* There are at least five microbial groups involved in anaerobic digestion:

1. Hydrolytic bacteria degrading polymers (polysaccharides, proteins, nucleic acids) to monomers (glucose, aminoacids, nucleosides).
2. Acidogenic bacteria fermenting monomers to organic acids and alcohols.
3. Acetogenic bacteria producing acetate from other organic acids and alcohols.
4. Acetotrophic methanogens, producing methane from acetate.
5. Hydrogenotrophic methanogens, producing methane from hydrogen and carbon dioxide.

If the cell concentration of these organisms per 1 mL is  $4 \times 10^7$ ,  $7 \times 10^8$ ,  $2 \times 10^7$ ,  $5 \times 10^8$  and  $1 \times 10^8$ , respectively, the Shannon-Weaver index ( $H$ ) of microbial diversity by physiological functions will be 13, and the evenness index ( $E$ ) will be 8.1. The diversity indices are related to the process efficiency and stability and can be used in environmental engineering to compare the processes with different operational parameters.

## 2.2. Interactions in Microbial Ecosystems

The types of interactions between the biotic elements of a microbial ecosystem (cells of microbial population, microbial populations, microorganisms and macroorganisms) are positive and negative. Positive interactions are as follows:

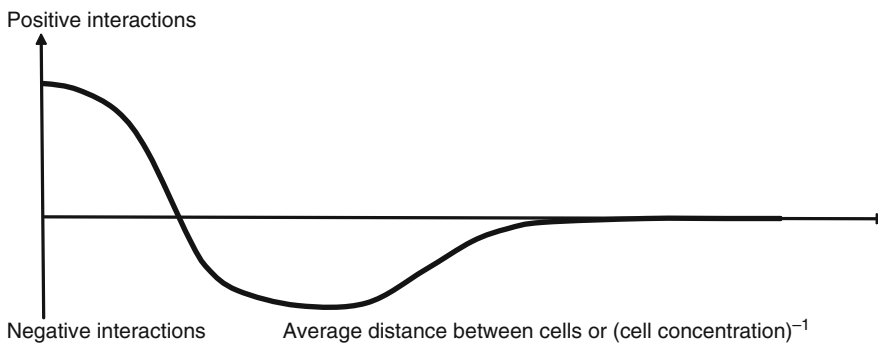
1. Commensalism (only one biotic element has benefits).
2. Cooperation, mutualism (both elements have benefits).
3. Essential mutualism, symbiosis (both elements cannot live separately).

Negative interactions are as follows:

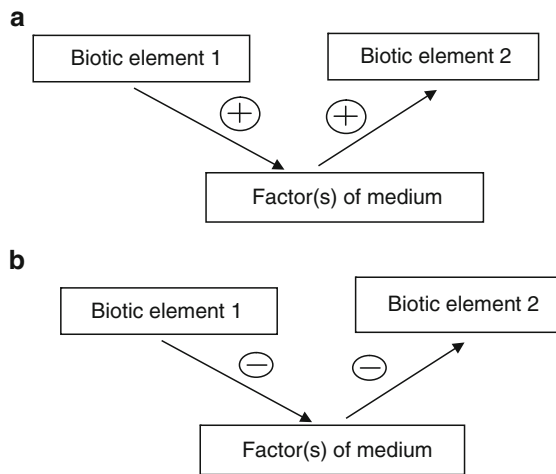
1. Neutral competition (organisms compete in the rate and efficiency of nutrients consumption, growth rate or in the resistance to unfavourable for growth environmental factors).
2. Antagonism (both abiotic elements suffer from interaction because they produce specific factors that negatively affect growth rate or other physiological or biochemical properties of competitors).
3. Amensalism (only one element suffers from the interaction).
4. Predation and parasitism; it is interaction when one element (prey) suffers and the other element (predator) benefits.

There may be neutralism, i.e., absence of positive or negative interactions between biotic elements.

The population density or average distance between biotic elements determines the type of interaction (Fig. 2.1).



**Fig. 2.1.** Microbial interactions depending on cell concentration in ecosystem or the distance between cells in community.



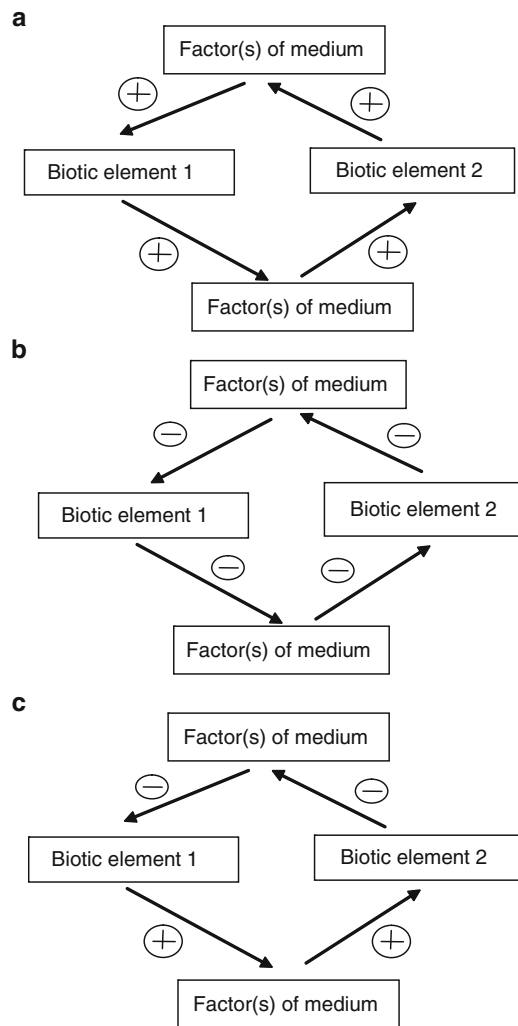
**Fig. 2.2.** Examples of microbial commensalism: (a), one group of microorganisms produces growth factor(s) essential for another group; (b), facultative anaerobes use oxygen and create anaerobic habitat suitable for the growth of obligate anaerobes.

When the population density is low, organisms have neither positive nor negative interactions. When the population density is medium, organisms compete among themselves for the availability of resources, by rate or efficiency of growth, and by production of metabolites, which negatively affect the growth of competitors. When the population density is high, cells usually aggregate and cooperate between themselves. Both competition and cooperation are carried out mainly because of the changes of chemical factors of environment such as concentration of nutrients, pH and redox potential of the medium, excretion of antibiotics, extracellular digestive enzymes, or heavy metals binding exopolysaccharides and simultaneous biodegradation of substances.

*Commensalism*, a microbial system relationship in which only one biotic element benefits, is realized by different ways (Fig. 2.2). There are thousands of examples of this interaction in environmental biotechnological systems. Some of them are as follows:

1. Facultative anaerobes use oxygen and create the conditions for the growth of obligate anaerobes; this interaction is important in the formation of anaerobic layer in microbial aggregates existing under aerobic conditions (6).
2. One group of microorganisms produces a growth factor essential for another group; this interaction is an obvious condition for the outbreak of pathogenic *Legionella pneumophila*, originated from such engineering systems as air conditioners, cooling towers and fountains.
3. Sequential biodegradation of xenobiotics by different groups of microorganisms; the microbial group performing biodegradation does not depend on the activity of the groups degrading its product of metabolism.

*Mutualism* is a type of interaction in which both biotic elements (microbial groups) have advantages from their interaction (Fig. 2.3).



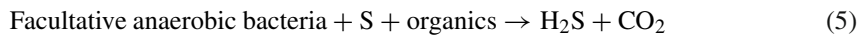
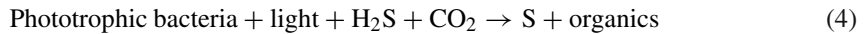
**Fig. 2.3.** Examples of mutual microbial interactions: **(a)**, both microbial groups are exchanging with growth factors or nutrients; **(b)**, both microbial groups diminish negative factors of medium; **(c)**, removal of oxygen by group 2 creates anaerobic conditions for fixation of nitrogen by group 1 which supplies nitrogen compounds for group 2.

Mutual interactions are facilitated by close physical proximity in microcolonies, biofilms and flocs. Physiological cooperation in biofilm or aggregates is supplemented and supported by its spatial structure, i.e., formation of microhabitats for individual populations. Some examples of mutualism are as follows:

1. Syntrophy (“co-eating”), both microbial groups supply growth factors or nutrients.
2. Sequential biodegradation of xenobiotics when the product of biodegradation inhibits biodegradation.



3. Cycling of element by two microbial groups:



4. Removal of oxygen by heterotrophic bacteria creates anaerobic conditions for fixation of nitrogen by phototrophic cyanobacteria, which supply nitrogen compounds for heterotrophic bacteria.

*Positive interactions between animals and microorganisms* are common and often essential for animals. Microorganisms can improve the digestion and assimilation of food by animals; produce growth factors, like vitamins and essential aminoacids for animals and keep out the pathogenic microorganisms from the surface and cavities of macroorganisms. For example, ants and termites cultivate cellulose-degrading fungi in their chambers to enhance the feeding value of plant material. Insects provide cellulose-degrading fungi with favorable conditions, and a supply of cellulose and mineral components. Another example is interactions between microorganisms and human organisms. The human body contains complex and stable microbial communities on the skin, hairs, body cavities, and within the gastrointestinal tract. Macroorganisms provide favorable conditions and supply nutrients to microorganisms, which produce some vitamins and keep out the pathogenic microorganisms from the skin and surface of cavities.

*Symbiotic mutualism*, or simply symbiosis, means that two groups cannot live separately. This is commonly the case in the interaction between macroorganisms and microorganisms. Many protozoa have symbiotic relations with bacteria and algae, often including them into the cell as endosymbionts. Bacterial endosymbionts supply the growth factors to the protozoan partner. A well-known example is the symbiosis of ruminant animals (cow, deer, sheep) and anaerobic, cellulose-degrading microorganisms in their rumen. Ruminant animals ensure crushed organics and mineral components, optimal pH and temperature, and microorganisms hydrolyze and transform cellulose to assimilated fatty acids.

*Positive interactions between plants and microorganisms* are common and often essential for plants. Epiphytic microorganisms live on aerial plant structures such as stems, leaves and fruits. The habitat and microorganisms on the plant leaves is called phyllosphere. The yeasts and lactic acid bacteria, for example, dominate in the phyllosphere. They receive carbohydrates and vitamins from the plant. High microbial activity occurs also in the soil surrounding the roots, called the rhizosphere. Organic compounds that stimulate heterotrophic microbes are excreted through the roots. Some fungi are integrated into the roots and contribute to plant mineral nutrition. This type of symbiotic interaction is called mycorrhizae. An example of mycorrhizae is the interaction between pine and fungi. Fungi integrated into the roots of pine contribute to plant mineral nutrition in exchange for a supply of organic nutrition from the plant.

*Symbiotic mutualism of plants and microorganisms* is a common interaction. A well-known example is the symbiotic fixation of atmospheric nitrogen, which is a major reservoir of nitrogen for life. The roots of some plants are invaded by nitrogen-fixing bacteria, mainly from the genus *Rhizobium*, which form tumor-like aggregates (nodule), where the bacteria are transformed into large cells (bacteroids) capable of fixing  $\text{N}_2$  from air. A plant supplies

the bacteroids with organic and mineral feed and the bacteroids supply organic nitrogen to the plant. Symbiotic relations ensure the existence of lichens where photosynthetic algae or cyanobacterial component of lichens produce organic matter and microscopic fungi provide mineral nutrient transport and the mechanical frame for the photosynthetic organisms. Most lichens are resistant to extreme temperatures and drying and are capable of fixing nitrogen and occupying hostile environments.

*Neutral competition* between the biotic elements (organisms/populations/groups) means competition by the rate of nutrients consumption or growth rate. There may also be neutral competition by affinity with the nutrients or by resistance to environmental factors unfavorable for growth. It is the most typical interaction between aquatic natural ecosystems and wastewater treatment engineering systems.

*Amensalism* is an active competition in which one biotic element produces a substance that inhibits the growth of another biotic element. There may be, for example, changes in pH caused by the production of inorganic and organic acids by one population. Neutral competition and amensalism are the main mechanisms for forming an enrichment culture where one or some species dominate after cultivation of an environmental sample. The production of antibiotics is a specific application of amensalism because antibiotic is a substance able to, at low concentrations, negatively affect the growth of sensitive cells. Antibiotic-producing microorganisms dominate in rich environments with optimal conditions for growth, i.e., in the biotops where neutral competition is not sufficient to ensure domination of one biotic element. These biotops are soil, phyllosphere, skin or cavities of animals, but not aquatic biotops with a low concentration of nutrients.

*Antagonism* is the active competition between two biotic elements, i.e., competition enhanced by specific tools such as excretion of chemical substances, including antibiotics, by two competing biotic elements.

*Predation* occurs when one organism engulfs and digests another organism. A typical predator-prey relationship exists between predator protozoa and bacteria. Therefore, the predator protozoa improve the bacteriological quality of the effluent after aerobic wastewater treatment because it helps to reduce the number of free-living bacteria.

*Parasitism* is a very common interaction between microorganisms and macroorganisms, and between different microorganisms. The benefiting parasite derives its nutritional requirements from the host, which is the harmed organism. All viruses are parasites of bacteria, fungi, algae, plants and animals. Some prokaryotes are parasites of prokaryotes. For example, *Bdellovibrio spp.*, small curved cells, are parasites of Gram-negative bacteria, and *Vampirococcus spp.* sucks the cytoplasm out of another bacterium. Enumeration of microbial parasites in the environmental sample by the zones of lysis on a Petri dish with a layer of specific bacteria is the simplest nondirect way to evaluate the pollution of environment with these bacteria. Growth of bacterial viruses (bacteriophages) can deteriorate the industrial cultivation process because of spontaneous lyses of bacterial cells. Bacteriophages are widely used in genetic engineering of bacterial strains as the vector for transfer of defined genes into bacterial cells.

Plant parasites are represented by phytopathogenic viruses, prokaryotes and fungi. These parasites cause plant diseases. Typical stages of disease are as follows:

1. Contact of the microorganism with the plant.
2. Entry of the pathogen into the plant.
3. Growth of the infecting microorganism.
4. Development of plant disease symptoms.

Microbial pathogens disrupt normal plant functions by producing enzymes, toxins and growth regulators. Some plant pathogens such as white-rot fungi or bacteria from genus *Pseudomonas* can degrade xenobiotics and are widely used in environmental engineering. Therefore, the risk of plant infection must be accounted for in environmental biotechnology operations, especially during soil bioremediation.

*Parasites of human and animals* are represented by pathogenic viruses, prokaryotes, fungi or protozoa. The pathogenic (infectious) microorganisms grow in animal tissue and can cause diseases in macroorganisms. Saprophytic microorganisms feed on dead organic matter. Opportunistic pathogens are normally harmless but have the potential to be pathogens for debilitated or immunocompromised organisms.

*Infection* refers to the disease transmission caused by the transfer of pathogenic microorganisms from the environment or from one macroorganism to another. Infectious microorganisms can enter a human through direct contact between individuals or reservoir-to-person contact. The diseases may be conventionally distinguished as air-borne, water-borne, soil-borne and food-borne infectious diseases. When infectious agents are spread by an insect such as a mosquito, flea, lice, biting fly or tick, they are referred to as vectors.

*Infectious diseases* still account for 30–50% of deaths in developing countries because of poor sanitation. By comparison, mortality from infectious diseases is 10 times smaller in developed countries. Transmission of water-borne diseases is directly related to the bacteriological quality of water and effluent of wastewater treatment plants. Sources of pathogens other than sewage outlets are wildlife watersheds, farms and landfills. The prevention of outbreaks of water-borne and air-borne diseases is one of the main goals of environmental biotechnology. Environmental engineers and epidemiologists must work closely to identify the reason of outbreak, find its source (reservoir), define the major means of transmission of infectious microorganisms, and to develop a way to stop or diminish the scale of outbreak.

*Factors of pathogenicity* include the following abilities of microorganisms:

1. Production of exotoxins, which are extracellular proteins. In this case, host damage can occur at sites far removed from a localized focus of infection. For example, anaerobic bacteria *Clostridia tetani* can be introduced from the soil into the body with deep puncture wounds. If the wound becomes anaerobic, the microorganism can grow and release its tetanus toxin, which causes spastic paralysis.
2. Production of enterotoxins, which are the exotoxins that act in the small intestine. These cause diarrhea, the secretion of fluid into the intestinal passage.
3. Production of endotoxins, which are lipopolysaccharides of the outer membrane of Gram-negative bacteria. Endotoxins are less toxic than exotoxins.
4. Formation of microstructures (fimbriae, flagellum) and macromolecules for specific adherence of microbial cells or viruses to a host cell.
5. Formation of cell structures (capsule) and macromolecules (O-antigen) protecting microbial cells from the reaction of a host macroorganism.

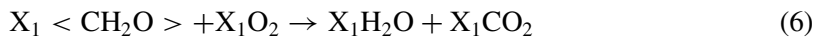
*Water-borne pathogens* enter the host body by ingestion of cells, cysts or viral particles. The most common water-borne pathogenic bacteria are pathogenic strains of *Escherichia coli*, *Leptospira spp.*, *Vibrio cholera*, *Shigella spp.*, *Salmonella spp.*, and *Campylobacter spp.* Two protozoans of major concern as water-borne pathogens are *Giardia intestinalis*, *Cryptosporidium spp.* and *Entamoeba histolytica*. One to ten ingested cysts of *Giardia* can cause diarrhea. There are over 100 known water-borne human enteric viruses.

An *indicator microorganism* is a conventionally selected microorganism or group of microorganisms used to determine the risk of water-borne infection associated with fecal contamination of water from humans or animals. There is a great variety of pathogenic organisms in water, and detection of each one in order to monitor water quality is an expensive operation. An indicator microorganism must be of the same origin and have similar physiological properties as some group of pathogens, and can be easily detected or enumerated in a water sample. Common indicators of water pollution with enteropathogens (main agents of water-borne diseases) from feces of warm-blooded animals are the numbers of the cells of *E. coli* (fecal coliforms), some *Streptococcus spp.* (fecal streptococci) or anaerobic *Clostridium spp.* The concentration of coliforms is usually less than 1 cell/mL in treated drinking water and more than some million ( $10^6$ ) cells/mL in sewage. The concentration of heterotrophic bacteria in water determined by heterotrophic plate count (HPC) is also important bacteriological parameter of water quality. The concentration of anaerobic bacteria from genera *Clostridium*, *Bifidobacterium* or *Bacteroides* may be considered a good indicator of fecal pollution of water because their content in feces in some orders larger than the content of coliforms. There are no indicator organisms for protozoan cysts and viruses because of the specific release and survival of every strain.

### 3. MICROBIAL GROWTH AND DEATH

#### 3.1. Nutrients and Media

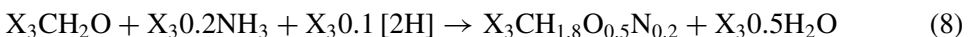
The *elemental composition* of biomass can be shown approximately by the formula  $\text{CH}_{1.8}\text{O}_{0.5}\text{N}_{0.2}$ . The average content of carbon in a microbial biomass is approximately 50%. The exact elemental composition can be determined by an automatic COHN analyzer and used in the design of the biotechnological process. For example, the aerobic growth of biomass ( $\text{CH}_{1.8}\text{O}_{0.5}\text{N}_{0.2}$ ) and biodegradation of carbohydrates shown by formula  $\text{CH}_2\text{O}$  can be described by the following equations:



which shows oxidation of carbohydrates to generate energy used for growth;



which shows oxidation of carbohydrates to generate reducing equivalents [2H] used for biomass synthesis; and



which shows assimilation of carbon from carbohydrates to biomass.

To ensure supply of all essentials for biological activity elements, a medium must contain so-called macronutrients (C, H, O, N, P, S, K, Na, Mg, Ca and Fe) and micronutrients (Cr, Co, Cu, Mn, Mo, Ni, Se, W, V and Zn). The recommended media for specific groups of microorganisms are given in numerous microbiological manuals.

The *chemical content* of microbial biomass varies moderately. The water content in microbial cells is 70–80%. The average content of dry matter in bacterial cells is as follows: protein, 55%; RNA, 15%; polysaccharides, 10%; lipids, 5%; DNA, 5%; monomers and inorganic ions, 10%. The quantity of DNA per cell is stable; quantities of RNA and protein per cell are larger at higher growth rate. Cells of algae or fungi grow at a lower rate than the majority of prokaryotes and include a larger quantity of cell wall material such as cellulose, silica and calcium carbonate. Therefore, the content of RNA and protein in biomass of fungi and algae may be lower than that of bacterial biomass.

“*Storage*” compounds. Quantities of “storage” compounds, which accumulate in cells in a rich medium under unfavorable conditions for growth, vary in a wide range. For example, the content of polyhydroxybutyrate (PHB), which accumulates in bacterial cells under an excess of carbon source and oxygen limitation, can reach 80% of dry mass. Other “storage” compounds are such intracellular polysaccharides as glycogen (for prokaryotes and eukaryotes), starches (for eukaryotes), extracellular polysaccharides (for prokaryotes), storage lipids (for eukaryotes), polyphosphate and sulphur granules in prokaryotes. Therefore, the content of any component ( $c_i$ ) of microbial biomass may be described by the following function:

$$(c_i) = (Ci) / [C_1 + C_2f_1(\mu) + C_3f_2(M)] \quad (9)$$

where  $C_i$  is the average quantity of  $i$ -component in cell,  $C_1$  is average quantity of components in cell (DNA, material of cell wall), which weakly depend on growth rate and medium conditions;  $C_2$  is the average quantity of components (RNA, protein) in cell, which significantly depend on specific growth rate ( $\mu$ );  $C_3$  is the average quantity of “storage” components in cell, which significantly depend on medium conditions (M).

*Medium content* must reflect the needs in elements and substances for desired microbial activity and growth. It can be defined medium, which is a mixture of pure mineral salts and organic substances or so-called complex medium containing organic and inorganic substances due to the digestion or extraction of natural ingredients such as meat, plant biomass, manure and food waste. Medium may be in liquid, solid, gaseous states or in their combinations. Microbial cultivation in laboratory is often performed on the surface of a solid gel medium.

*Carbon sources for growth.* Prototrophic microorganisms can use one source of carbon and energy to synthesize all organic components of a cell. Auxotrophic microorganisms require supply of growth factors, i.e., some components of biomass, such as vitamins, aminoacids, nucleosides or some fatty acids from the medium. They lose their ability to synthesize these substances due to presence of these substances in their natural habitats. Autotrophic microorganisms can use only  $\text{CO}_2$  to synthesize all organic components of the cell. Heterotrophic microorganisms use organic sources of carbon to synthesize cell biomass.

### 3.2. Growth of Individual Cells

Growth, proliferation and differentiation. Growth is defined as an increase in individual cell mass or the mass of cell population. Proliferation (cell division) is defined as an increase in cell number. Cell proliferation by binary fission is the most common type. However, there may be “unbalanced” proliferation, which consists of multiple fissions of cells without their growth. There may be also “unbalanced” growth without proliferation. This appears as elongation or enlargement of cells without their divisions. Cell differentiation is the transformation of microbial cells into specialized cells. Examples of such cells are as follows:

1. An endospore is an anabiotic (i.e., temporarily not active) cell with low content of water and covered by thick envelope, serving for survival under unfavorable conditions for growth, e.g., starvation, dry environment and high temperature.
2. An exospore is similar to an endospore by its properties, but not forming in the mother cell; the main functions of these cells are to increase survival and dispersion of cells in the environment.
3. An anabiotic cyst is an enlarged cell with the main function of increased survival.
4. Nitrogen-fixing cysts and bacteroides are enlarged cells whose main function is the transformation of atmospheric nitrogen into aminogroups of organic substances.

*Eukaryotic cell cycle.* There is strict coordination of a cycle of individual cell growth and division with DNA replication cycle in a eukaryotic cell. A eukaryotic cell cycle (mitotic cycle) has the following phases:

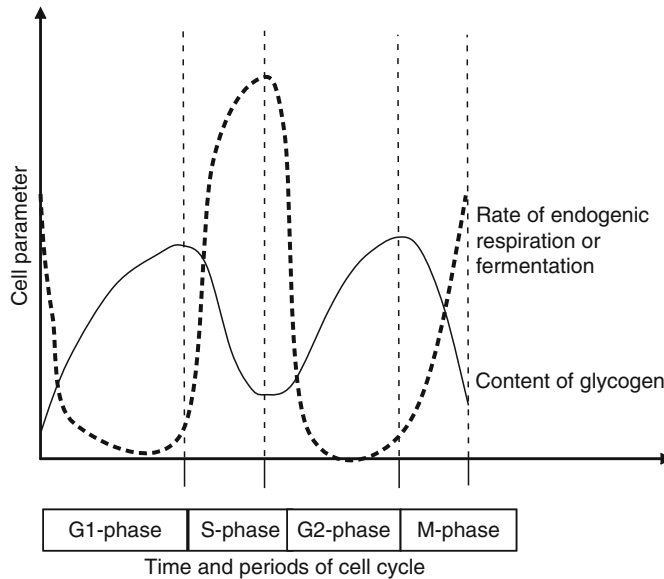
1. G<sub>1</sub>-phase is a period between cell division and initiation of DNA replication; the duration of mitotic cycle is usually proportional to the duration of G<sub>1</sub>-phase; differentiation of cells starts from G<sub>1</sub>-phase.
2. S-phase is a period of chromosomal DNA replication.
3. G<sub>2</sub>-phase is a period between termination of DNA replication and mytosis (splitting of nucleus).
4. M-phase is a period of mytosis, splitting of nucleus.

*Prokaryotic cell cycle.* There is certain coordination between the cell division cycle (period between consecutive cell divisions) and DNA replication cycle (period between initiation and termination of chromosomal DNA replication) in a prokaryotic cell. However, this coordination is not as strong as in eukaryotes. Depending on growth or proliferation rates, there may be some cycles of DNA replication within a cell division cycle, or even a cell division cycle without a DNA replication cycle accompanying the formation of DNA-free daughter cells (7).

*Coordination of cell cycle events.* There are many levels of coordination between biochemical and physiological cell activities during a cell cycle:

1. Individual RNAs and enzymes synthesis and degradation.
2. Regulation of enzyme activity by metabolites and co-factors.
3. Regulation of catabolism and energy storage.
4. Regulation of whole-cell activity by different cell regulators.

*Periods of exotrophy and endotrophy in cell cycle.* A simple theory explaining coordination of cell cycle events is alternation of the periods of exotrophy and endotrophy in cell cycle (8). It was demonstrated experimentally that G<sub>1</sub>- and G<sub>2</sub>- phases of mitotic cycle comprise the phases of exotrophy when the external source of carbon and energy is extensively transformed



**Fig. 2.4.** Example of alterations of exo- and endotrophy in cell cycle of eukaryotes.

into energy and carbon store (glycogen, starch, lipids). S- and M-phases, which are the most sensitive periods of a cell cycle, are the phases of endotrophy. During endotrophy, the accumulated store of energy and carbon is utilized for DNA replication and mytosis, respectively (Fig. 2.4).

External sources of energy and carbon are not assimilated during these periods. The alternations between the periods of exotrophy and endotrophy are performed due to the increase or decrease of intracellular concentration of cyclic AMP and are accompanied by alternation of the charge of membrane potential (proton-motive force).

*Different sensitivity of cells in the states of exo-and endotrophy.* Environmental factors, which are unfavorable for DNA replication, retain cells in phases of endotrophy, G<sub>1</sub>- or G<sub>2</sub>-phases. An extended period of exotrophy leads to enormous intracellular accumulation of carbon and energy sources (8). Exotrophic and endotrophic cells are distinguished by their biochemical and physiological properties so greatly that it would be useful to study these two different groups of cells.

*Cell age and cell trophic state distributions in microbial population.* Due to asynchronous cell cycles of individual microbial cells, there is a distribution of cells with different ages and cell trophic states in population. Cell size, DNA content, and percentage of exotrophic or endotrophic cells can be used to monitor cell population by flow cytometry. For example, duration of exotrophy ( $\Delta t_{\text{ex}}$ ) and duration of G<sub>1</sub>-phase ( $\Delta t_{\text{G}_1}$ ) of yeasts are linearly related to the duration of cell cycle ( $T$ ) (7, 8):

$$\Delta t_{\text{ex}} = 0.5 T - 1.0 \quad (10)$$

and

$$\Delta t_{G1} = 0.7 T - 0.9 \quad (11)$$

Using these equations, the specific growth rate in population ( $\mu$ ) can be determined from the microscopic view or flow cytometry distribution, taking into account that  $T = \ln 2/\mu$ . G<sub>1</sub>-cells of the budding yeasts can be determined as cells without buds. Exotrophic and endotrophic cells of bacteria can be also distinguished after adding a small quantity of cooxidizing substrate, producing toxic products of oxidation. For example, allyl or amyl alcohol can be added to cells that utilize ethanol. As a result, cells will produce allyl or amyl aldehyde, which cannot be further oxidized and therefore, kill cells. Exotrophic cells die after this incubation but endotrophic cells remain alive because they do not consume and oxidize external sources of carbon and energy. The share of exotrophic cells increases during starvation and other unfavorable conditions because the S-phase cannot be started until intracellular accumulation of sufficient quantity of carbon and energy sources is finished (8).

### 3.3. Growth of Population

*Exponential growth and proliferation.* Growth is an increase of biomass concentration or content ( $X$ ). Proliferation is an increase in cell concentration or content ( $N$ ). The balanced growth of microorganisms is followed by a proportional increase of cell number and biomass in the studied system. Under unfavorable conditions, the proliferation can be stopped, but not growth, resulting in long or large cells in the population. Under some conditions, these large cells will stop growing and split into smaller cells. The time required to form two new cells from one cell during the balanced growth is generation time,  $t_g$ . Exponential and balanced proliferation and growth are described by the equations:

$$N = N_0 2^n \quad (12)$$

or

$$X = X_0 2^n \quad (13)$$

where  $N_0$  is the initial cell concentration,  $n$  is the number of generations,  $X$  is concentration of the biomass after  $n$  generations,  $X_0$  is initial concentration of biomass. Exponential growth is conventionally described by the equations:

$$dX/dt = \mu X \quad (14)$$

or

$$\mu = (\ln X - \ln X_0)/t, \quad (15)$$

where  $\mu$  is specific growth rate and  $t$  is the duration of exponential growth. Typical specific growth rates are from 0.2 to 1.0/h<sup>-1</sup> for unlimited growth of heterotrophic bacteria and from 0.01 to 0.2/h<sup>-1</sup> for the growth of microscopic fungi. In such specific cases as apical (on the tip) elongation of thread-like, nonbranching hypha of mycelial fungi or actinomycetes, growth can be described by a linear equation.



Growth efficiency is determined by a growth yield that is a ratio between quantity of produced biomass and consumed nutrient ( $Y_{X/S}$ ) or energy ( $Y_{X/E}$ ).

For the batch system, it is determined by the equation:

$$Y_{X/S} = (X_t - X_0)/(S_0 - S_t) \quad (16)$$

where  $S_t$  is substrate concentration in the system at the end of period  $t$ ,  $S_0$  is the initial substrate concentration. Growth yield ( $Y_{X/S}$ ) for the continuous system without recycling of the biomass is determined by the following equation:

$$Y_{X/S} = X/(S_i - S_e) \quad (17)$$

where  $X$  is a biomass concentration,  $S_i$  and  $S_e$  are the concentrations of substrate in the influent and effluent.

*Microbial batch culture.* A semiclosed system of cultivation is called a batch culture. There is usually a supply of air, release of gaseous products and additions of titrant and antifoam substance during this type of cultivation. Due to exhaustion of nutrients and accumulation of biomass and metabolites, the sequence of following phases is typical in batch culture:

1. Lag-phase or phase of cells adaptation and self-control of environment; its duration depends on the concentration of inoculated biomass and magnitude of the difference between previous and current conditions of cultivation.
2. Short log-phase or phase of exponential growth.
3. Transitional period between log-phase and stationary phase.
4. Stationary phase is characterized by slow growth or its absence due to exhaustion of nutrients or accumulation of metabolites.
5. Death phase is characterized by an increasing number of dead cells and their lysis.

Microbial continuous culture is open for exchange by gases and liquids. There is a large diversity of aerobic and anaerobic bioreactors for continuous cultivation, for example:

1. Bioreactors of complete mixing; the most common type is a chemostat where the dilution rate ( $D$ ), which is a ratio between flow rate ( $F$ ) and working volume of the reactor ( $V$ ), is maintained constant.
2. Plug-flow systems.
3. Consecutively connected bioreactors of complete mixing, which form a plug-flow system.
4. Fixed biofilm reactor or retained biomass reactor with the flow of medium through it; biomass is retained in the reactor due to adhesion, sedimentation or membrane filtration.
5. Complete mixing or plug-flow continuous cultivation with recycling of microbial biomass.
6. Semicontinuous and sequencing batch cultivation, which is a continuous cultivation with the periodical addition of nutrients and removal of suspension.

### 3.4. Effect of Environment on Growth and Microbial Activities

*Macro- and microenvironments.* There is no gradient of parameters that depends on activity of microorganisms in a macroenvironment; however, there is a gradient of parameters depending on the microbial activity in a microenvironment. The typical scale of such microbial microenvironments as aggregate, biofilm, or microbial mat is between 0.1 and 100 mm. An artificial microenvironment is created due to adhesion of cells on carrier or cell incorporation

into carrier. Cells can be concentrated not only on liquid–solid interphase but also on liquid–gas interphase because the nutrient concentrations are higher there than in the bulk of liquid. A medium (pl. *media*) is an artificial environment for the cultivation of microorganisms in the form of solution, suspension or solid matter.

*Effects of nutrients on growth rate.* Kinetic limitation means that specific growth rate depends on the concentration of limiting nutrient. Usually, it is one limiting nutrient, but there may be simultaneous limitation by some nutrients. If one nutrient limits the specific growth rate, this dependency is often expressed by Monod's equation:

$$\mu = \mu_{\max} [S/(S + K_s)] \quad (18)$$

where  $\mu_{\max}$  is the maximum of specific growth rate;  $S$  is concentration of the nutrient (substrate), limiting growth rate;  $K_s$  is a constant. However, there are hundreds of other known models describing  $\mu$  as  $f(S_i)$ . A double limitation of  $\mu$  by donor of electrons and oxygen is typical for the cases when the initial step of catabolism is catalyzed by oxidase or oxygenase incorporating atom(s) of oxygen into a carbon molecule or energy source.

*Effects of nutrients on yield.* The stoichiometric limitation of growth means that the dosage of the nutrient in the medium linearly determines the yield of biomass. For some groups of prokaryotes, the sources of carbon and energy are separated. Growth efficiency depends on energy extracted during catabolism of the energy source. Growth yield reflects the balance of energy produced in catabolism and energy assimilated in biosynthesis. Typically, in microbial growth there is no feedback regulation between the rates of biosynthesis and catabolism. Therefore, limitation of biosynthesis due to nutrient limitation or unfavorable physical factors of environment diminishes growth yield. However, there may be paradoxical increase of growth yield under unbalanced biosynthesis and catabolism and excess of carbon source. This can be caused by the redirection of carbon flow under excess of carbon source to the synthesis of storage carbohydrates or PHB, which require less energy for their synthesis than cell biomass. Another portion of energy, in the case of unbalanced catabolism and biosynthesis, can be used for the synthesis of extracellular polysaccharides or intracellular accumulation of polyphosphates, polypeptides or low molecular weight osmoprotectors.

*Effect of starvation on microorganisms.* There are three typical responses of microorganisms to starvation, i.e., shortage of some nutrients in a medium. The bacteria known as R-tactics are fast growing in a rich medium but can quickly die under a shortage of nutrients. Typical representatives of this group are *Pseudomonas spp.* The L-tactics bacteria are fast growing in a rich medium, but under starvation, they form dormant spores and cysts. Typical representatives of this group are *Bacillus spp.* K-tactics bacteria are adapted to grow slowly in a medium with a low concentration of nutrients. Typical representatives are the oligotrophes, *Hyphomicrobium spp.*

*Effect of oxygen on growth.* Aerobes are microorganisms that grow at the atmospheric pressure of oxygen (0.21 atm). Microaerophiles prefer a low concentration of oxygen because they have oxygen-sensitive molecules. Aerotolerant anaerobes have no need for oxygen to grow but can tolerate its presence in the medium. Obligate (strict) anaerobes are sensitive to oxygen because they have no protection against such toxic products of oxygen reduction as hydrogen peroxide ( $H_2O_2$ ), superoxide radical ( $O_2^-$ ) and hydroxyl radical ( $OH\cdot$ ). The

relationship to oxygen can be easily determined in the laboratory and is one of the most important identification properties of microorganisms because it was created in microbial evolution in parallel with the planet's evolution from an anaerobic to an aerobic atmosphere. The concentration of dissolved oxygen for the specific growth rate of aerobic microorganisms is usually limited to below 0.1 mg/L, but in the cases where the initial step of catabolism is catalyzed by oxidase or oxygenase, it can be significantly higher, up to 1 mg/L.

*Effect of temperature on growth.* The maximum temperature for growth depends on the thermal sensitivity of secondary and tertiary structures of proteins and nucleic acids. The minimum temperature depends mainly on the freezing temperature of the lipid membrane. The optimal temperature is close to the maximum temperature. Different physiological groups of microorganisms adapt to different temperatures. Psychrophiles have optimal temperatures for growth below 15°C. Mesophiles have optimal growth temperatures in the range between 20 and 40°C. Thermophiles grow best between 50 and 70°C. There are known thermoextremophiles growing at temperatures higher than 70°C.

*Effect of pH on growth.* Natural biotops have differing pH values: pH 1–3 (gastric juice, volcanic soil, mine drainage); pH 3–5 (plant juices, acid soils); pH 7–8 (fresh and sea water); pH 9–11 (alkaline soils and lakes). Acidophiles grow at pH lower than 5, neutrophiles grow within the pH range from 5.5 to 8.5, and alkalophiles grow at pH higher than 9. Intracellular pH is an approximately neutral pH. Extracellular pH affects the dissociation of carboxylic-, phosphate-, and amino-groups of a cell's surface, thus changing its charge and adhesive properties. This feature is important for the sedimentation of activated sludge, cell aggregation, and formation of microbial biofilm.

*Effect of osmotic pressure on growth.* The majority of microorganisms can live with a concentration of salts in the medium up to 30 g/L. A higher concentration of salts or organic substances can cause water to diffuse out of the cell by osmosis. However, some groups of microorganisms adapt to high osmotic pressure or low activity of water. These halophiles require the addition of NaCl in the medium during their isolation and cultivation. Extreme halophiles require a high concentration of NaCl (15–30%) in the medium. Xerophiles are able to live in a dry environment. The main adaptation characteristic of halophiles and xerophiles is their ability for intracellular accumulation of such low molecular weight hydrophilic osmoprotectors as polyols, oligosaccharides and aminoacids.

### 3.5. Death of Microorganisms

*Natural death of microorganisms.* The equally splitting cells of bacteria are considered almost immortal creatures. However, some bacterial cells die even in pure culture at optimal growth conditions. Hypothetically, the death of microorganisms at optimal conditions for growth can be caused by a small asymmetry always determined in cell division and accumulation or depletion of asymmetrically separated cell components. The asymmetry of the division of eukaryotic cells is visible so that mother and daughter cells can be often distinguished. There may be accumulation or depletion of inert or essential cell components in this asymmetrical division, budding or splitting of eukaryotic cells. Therefore, the percentage of dead cells in the population of pure eukaryotic culture under optimal conditions for growth can be from 1 to 5%. Other reasons for natural cell death may include the production of

toxic oxygen radicals, shortage of essential cell components due to starvation, changes in structure of cell biopolymers and lipid components due to unfavorable physical parameters of the microenvironment.

*Fate of released microorganisms in environment.* The natural death of pathogenic microorganisms released to the environment is the most important factor in the termination of infectious diseases outbreaks. Active biodegraders, which are used in environmental engineering, are often opportunistic pathogens or genetically modified strains. Therefore, the study of environmental fate, death rate and survivability of microorganisms, which are used in bioremediation of polluted soil or spills in marine environment and released to environment, is essential for determining process feasibility. A rule of the thumb is that applied microorganisms must have some reasonable limits of lifetime in the treated and surrounding areas. This short lifetime can prevent accumulation or spread of unwanted microorganisms in the environment.

*Control of microbial death.* The control of unwanted microbial growth can be performed by physical or chemical inhibition of growth, killing of the microorganisms or their removal from the environment. Antimicrobial agents kill cells of bacteria, fungi or inactivate viral particles; thus, the terms bactericidal, fungicidal and viricidal agents are used. The most sensitive targets of the microbial cell are as follows:

1. Integrity of cytoplasmic membrane.
2. Active centers of enzymes.
3. Secondary, tertiary and quaternary structures of enzymes.
4. Primary and secondary structures of nucleic acids.

*Heat treatment.* The rate of cell death under heat treatment is a function of the first order:

$$dX_d/dt = k(X_0 - X_d) \quad (19)$$

or

$$\ln(1 - X_d/X_0) = -kt \quad (20)$$

where  $X_d$  and  $X_0$  are the numbers of dead cells and initial number of alive cells, respectively;  $t$  is the time of exposure, and  $k$  is a constant of decay. Another parameter, the decimal reduction time ( $D$ ), which is the time required for tenfold reduction of the population, is used in practice:

$$D = (\ln 0.1)/(-k) = 2.3/k \quad (21)$$

Pasteurization kills the vegetative cells of bacteria, fungi and protozoa. Vegetative cells of bacteria have a decimal reduction time in the range of 0.1–0.5 min under a temperature of 65°C. During bulk pasteurization, the liquid is exposed at 65°C for 30 min. During flash pasteurization, the liquid is heated to 71°C for 15 s and then rapidly cooled. Pasteurization reduces the level of microorganisms in the treated staff but does not kill all of them. Heat sterilization kills all microorganisms in the treated staff. It is often performed in autoclave. Bacterial endospores cannot be killed at the temperature of boiling water. Therefore, the autoclave uses steam under the pressure of 1.1 atm, which corresponds to a temperature of 121°C. The time of exposure of the sterilized material in autoclave under 121°C must be from 10 to 20 min.

*Sterilization by radiation.* Electromagnetic irradiation such as microwaves, ultraviolet (UV) radiation, X-rays, gamma rays and electrons are also used to sterilize materials. UV

irradiation, which does not penetrate solid, opaque or light absorbing materials, is useful for disinfection of the surfaces, air, and water. Gamma and X-rays, which are more penetrating, are used in the sterilization of heat-sensitive materials, especially biomedical plastics.

*Sterilization by filtration* of cells and viruses is performed in the bulk of filtration material by using particles adsorption or polymer membranes with a defined diameter of pores. Depth filters consist of a random array of overlapping fibers. Depth filters adsorb particles on the fiber surface. Membrane filters contain a large number of pores with a diameter smaller than cell size. Thus, microorganisms are trapped on the surface of the membrane. Sterilization of liquids by filtration through the membrane preserves sensitive substances, usually biological polymers, antibiotics and vitamins that are easily inactivated by heat. Bulk filtration (adsorption) is used mainly for the removal of fungi spores, bacterial cells and virus particles from air. Filtration, coagulation and settling processes, which are used in the treatment of drinking water, help reduce and remove pathogens with the sediments and consumption of particles by protozoa living on the surface of filtration material.

*Conservation* (preservation) refers to the prevention of microbial spoilage of organic materials by the following means:

1. Lowering the storage temperature.
2. Lowering pH.
3. Drying.
4. Addition of salt or organic substances to decrease water activity.
5. Addition of organic solvent (ethanol).
6. Formation of anaerobic conditions.

*Disinfectants and antiseptics.* Disinfectants such as chlorine gas, chloramine, ozone and quaternary ammonium compounds are chemical antimicrobial agents that are used on inanimate objects. Antiseptics such as iodine, 3% solution of hydrogen peroxide and 70% solution of ethanol, are chemical antimicrobial agents that are used on living tissue. Antimicrobial activity can be characterized by minimum inhibitory concentration (MIC), which is the amount of antimicrobial agent required to inhibit the growth of the test organism. MIC may be determined by the tube dilution technique or by the agar diffusion method.

Disinfectants and antiseptics participate in the following modes of action:

1. Destruction of integrity of cell membrane by organic solvents, for example, ethanol, acetone and hexane.
2. Destruction of integrity of cell membrane by anionic, cationic and nonpolar surfactants (detergents).
3. Destruction of active centers of enzymes by oxidants (iodine, chlorine, ozone) and heavy metals (Hg, Cu, Ag).
4. Destruction of structure of proteins and nucleic acids by oxidants (iodine, chlorine, ozone), heavy metals (Hg, Cu, Ag) and organic solvents (ethanol, phenols).

*Antibiotics* are microbial or chemically synthesized substances that are used to treat infectious diseases because of their ability to inhibit specific microbial species. The mode of action is specific for each thermophiles grow best antibiotic and is based usually on the inhibition of specific enzyme activity or inactivation of an active center of specific enzyme. There are

thousands of known antibiotics but only some hundreds are applicable in medicine because of the toxic effects of antibiotics on humans. Antibiotics are used in environmental biotechnology to select specific strains of microorganisms and in the construction of recombinant strains.

*Disinfection* is the chemical or physical treatment of water or wastewater treatment plant effluent by strong oxidants such as chlorine, chloramines, chlorine dioxide, ozone, ferrate or by UV, with the aim to diminish the concentration of defined microorganisms and viruses to some level.

The rate of cell death ideally should follow first-order kinetics:

$$dX/dt = -kt \quad (22)$$

or

$$\ln(X/X_0) = -kt \quad (23)$$

where  $X$  and  $X_0$  are the final and initial numbers of living cells,  $t$  is time of exposure, and  $k$  is a constant of decay that depends on the conditions of the disinfection. However, in the actual kinetics of disinfection, the order of the equation and a constant of decay change during the disinfection process are due to the presence of microorganisms with different resistance levels to the disinfectant.

*Resistance of different groups of microbes to disinfection.* Generally, the resistance of microbes to disinfections follows this order: vegetative bacteria (most sensitive group) → viruses → spore-forming bacteria → protozoan cysts (most resistant). Test microorganisms can be used, in some cases, to study water disinfection kinetics and to compare different disinfectants and regimes of disinfection instead of the pathogens. Indicator microorganisms are used often as test microorganisms. For example, cells of *Escherichia coli* can be used as test organisms in the disinfection of media containing pathogenic enterobacteria.

*Comparison of chemical disinfectants.* Disinfectants can be compared under the same conditions and microorganisms, by  $k$ , a constant of decay, or other technical or economical parameters. A benefit of chlorination is that chlorine residue remains in the water during distribution, which protects against recontamination. An undesirable side effect is that chlorination forms trihalomethanes, some of which are suspected carcinogens and bad-smelling chlorophenols. The efficiency of disinfection by chlorine gas decreases with pH because nondissociated hypochlorous acid (OHCl) is more active than hypochlorite ion (OCl<sup>-</sup>). Chloramines (NH<sub>2</sub>Cl and NHCl<sub>2</sub>) are weaker disinfectants than chlorine but are effective in the control of microbial biofilm with an exopolysaccharide matrix. Ozone is more effective against viruses and protozoa than chlorine, but it is more expensive because it is generated by electrical discharge in a dry air stream at the site of application. Another disadvantage is that there is no residual antimicrobial activity after ozonation. Therefore, ozonation and chlorination are commonly used in sequence.

*UV disinfection of water.* UV radiation damages microbial DNA at a wavelength of 260 nm. Microbial inactivation is proportional to the UV dose. Humic substances, phenolic compounds and suspended solids interfere with UV transmission. Microbial cells can be reactivated by repairing DNA damages with exposure to visible light. Therefore, UV-treated water should not be exposed to light during storage.

*Chemical and physical interferences with chemical disinfection.* Ferrous and manganese ions, nitrites, sulphides and organic substances reduce the concentration of oxidizing disinfectants, thus reducing the inactivation of microorganisms during disinfection. Therefore, these substances must be removed or preoxidized before disinfection. The particles of clay, silt, iron hydroxides, aggregation of cells, their encapsulation in slime, in macroorganisms, or coverage of microorganisms by the sheath, significantly reduce the inactivation of microorganisms and viruses during disinfection because of a steep gradient of oxidant in aggregates and its low concentration in cells. Therefore, particles and aggregates must be removed from water by coagulation and filtration or aggregation prior to disinfection.

## 4. DIVERSITY OF MICROORGANISMS

### 4.1. Physiological Groups of Microorganisms

*Evolution of prokaryotes and atmosphere.* According to geological data, the age of Earth is about  $4.6 \times 10^9$  years old. The first organisms, prokaryotes, appeared about  $3.5\text{--}3.8 \times 10^9$  years ago. There was no oxygen in that atmosphere, so the first organisms were anaerobes. The next step was an accumulation of atmospheric oxygen by oxygenic (oxygen-producing) phototrophic prokaryotes on the boundary  $2.2\text{--}2.0 \times 10^9$  years ago. Eukaryotes appeared about  $1.8\text{--}1.5 \times 10^9$  years ago, probably because of the intracellular symbiosis of smaller and bigger cells. The aerobic atmosphere led to the formation of the ozone barrier for intensive UV radiation on the Earth's surface. It was the primary condition for the creation of terrestrial life and multicellular organisms  $0.6\text{--}0.5 \times 10^9$  years ago.

*Diversity of energy generation types.* Due to their long-term evolution, microorganisms have two major mechanisms for biological energy generation and related physiological groups:

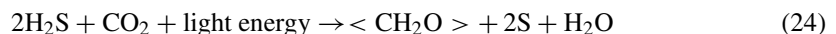
1. Chemotrophy is the generation of biologically available energy due to the oxidation and reduction of chemical substances.
2. Phototrophy is the generation of biologically available energy due to the capture and transformation of light energy.

There are two types of chemotrophy and related physiological groups:

1. Organotrophy (chemoorganotrophy to be exact) is the generation of biologically available energy due to the oxidation of organic substances.
2. Lithotrophy (lithochemotrophy to be exact) is the generation of biologically available energy due to the oxidation of inorganic substances, for example,  $\text{Fe}^{2+}$ ,  $\text{H}_2\text{S}$ ,  $\text{S}$ ,  $\text{NH}_4^+$ ,  $\text{NO}_2^-$ .

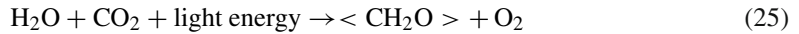
There are two types of phototrophy and related physiological groups of prokaryotes:

1. Anoxygenic photosynthesis is the generation of biologically available energy from light energy under anaerobic conditions, using sulphide as an electron donor:



where  $\langle \text{CH}_2\text{O} \rangle$  is the conventional formula of organic matter produced from  $\text{CO}_2$ .

2. Oxygenic photosynthesis is the generation of biologically available energy and oxygen from light energy, using oxygen of water as an electron donor:



*Diversity of energy generation by organotrophes.* There are different ways to generate biologically available energy by organotrophes and related physiological groups:

1. Fermenting organisms produce biologically available energy under anaerobic conditions (i.e., absence of oxygen and other acceptors of electrons) by fermentation, which is an intramolecular oxidation/reduction; one part of the molecule is oxidized and another part is simultaneously reduced.
2. Anaerobic respiring organisms produce biologically available energy under anoxic conditions (i.e., absence of oxygen but presence of other acceptors of electrons) by anaerobic respiration, i.e., oxidation of organic matter by acceptor of electrons other than oxygen, for example,  $\text{Fe}^{3+}$ ,  $\text{SO}_4^{2-}$ ,  $\text{CO}_2$ .
3. Aerobically respiring organisms produce biologically available energy under aerobic conditions (i.e., presence of oxygen) by aerobic respiration.

#### 4.2. *Phylogenetic Groups of Prokaryotes*

*Phylogenetic taxonomy* compares the gene sequences of macromolecules of homologous (similar) function from different species. The phylogenetic relationships between microbial groups have been determined by the comparison of rRNAs, part of ribosome, which is a conservative, slow-evolving cell component used for protein synthesis. Ribosomal RNA is often considered the best tool to infer prokaryotic phylogeny because it is one of the most constrained and ubiquitous molecules available, and thus, the most informative (9). The established branching order shows the three domains of life: Bacteria (Eubacteria), Archaea and Eucarya (Eukaryotes). The last domain includes the kingdoms of plants, animals, fungi and protists.

*Application of rRNA sequences in Microbiology of Environmental Engineering Systems.* The time scale of rRNA sequence changes can be expressed in terms of  $10^9$  years. The discrepancy between rRNA sequences, A and B, is conventionally called the evolutionary distance ( $E_{AB}$ ). It may be determined after computer or manual alignment as a number of the differences between the sequences (number of the mutations) per 100 sites (positions) of the compared sequences, i.e., as percentage of the mutations in compared sequences. The dissimilarity or similarity between the sequences of some organisms can be shown in a table (matrix), or in the form of a tree showing the hypothetical branching order of the organisms during their evolution. Different kinds of phylogenetic trees can be generated by computer analysis. The main applications of rRNA sequencing in environmental engineering are as follows:

1. The sequences of rRNA genes of strain or clone can be determined by the DNA sequencing machine, and the strain can be identified by comparison with known sequences downloaded from the databases.
2. The collections of rRNA gene sequences are used to study phylogenetic (evolutionary) relationships between microorganisms.



3. Specific parts of 16S rRNA sequences can be used as short (10–25-mer) labeled oligonucleotide probes for the detection of strains, genera, families or higher taxonomic units by the specific binding (hybridization) between target sequence rRNA and the probe.
4. Whole cell fluorescence in situ hybridization (FISH) with rRNA-targeted, fluorescent oligonucleotide probes is a popular approach to study the microbiology and spatial structure of complex microbial communities.

*Major rRNA Phylogenetic Divisions of Archaea* can be found in the classification of the Ribosomal Database Project (RDP-II) (10). The number of known sequences (data taken from <http://rdp.cme.msu.edu/>) reflects the abundance of the group and research interest to this group is shown in parentheses:

- [1] ARCHAEA (1173)
  - [1.1] EURYARCHAEOTA (822)
    - [1.1.1] METHANOCOCCALES (45)
    - [1.1.2] METHANOBACTERIALES (206)
    - [1.1.3] METHANOMICROBACTERIA AND RELATIVES (504)
    - [1.1.4] THERMOCOCCALES (66)
    - [1.1.5] METHANOPYRALES (1)
  - [1.2] CRENARCHAEOTA (351)
    - [1.2.1] THERMOPHILIC CRENARCHAEOTA (160)
    - [1.2.2] NONTHERMOPHILIC CRENARCHAEOTA (189)
    - [1.2.3] ENVIRONMENTAL CLONE PJP27 SUBGROUP (2)

*Major rRNA phylogenetic divisions of Bacteria* can be found in the classification of the Ribosomal Database Project (RDP-II) (10). The number of known sequences (data were taken from <http://rdp.cme.msu.edu/>) reflects the abundance of the group and research interest to this group is shown in parentheses:

- [2] BACTERIA (15104)
  - [2.1] THERMOPHILIC OXYGEN\_REDUCERS (42)
  - [2.2] THERMOTOGALES (33)
  - [2.3] CTM PROTEOLYTICUS\_GROUP (2)
  - [2.4] STRAIN EM 19 (1)
  - [2.5] ENVIRONMENTAL CLONE OPB45 GROUP (11)
  - [2.6] STR.SBR2095 (1)
  - [2.7] GREENNON-SULFUR BACTERIA AND RELATIVES (165)
  - [2.8] ENVIRONMENTAL CLONE OPB80 GROUP (14)
  - [2.9] LEPTOSPIRILLUM-NITROSPIRA (92)
  - [2.10] PROSTHECOBACTER\_GROUP (99)
  - [2.11] ANR.THERMOTERRENUM GROUP (29)
  - [2.12] ENVIRONMENTAL CLONE OPB2 GROUP (3)
  - [2.13] NITROSPINA\_SUBDIVISION (197)
  - [2.14] FLS SINUSARABICI ASSEMBLAGE (13)
  - [2.15] FLEXIBACTER-CYTOPHAGA-BACTEROIDES (781)

- [2.16] GREEN SULFUR BACTERIA (48)
- [2.17] ENVIRONMENTAL CLONE G37 GROUP (2)
- [2.18] ENVIRONMENTAL CLONE WCHB1–31 GROUP (52)
- [2.19] ENVIRONMENTAL CLONE UN104 GROUP (8)
- [2.20] PLANCTOMYCES AND RELATIVES (281)
- [2.21] CYANOBACTERIA AND CHLOROPLASTS (523)
- [2.22] ENVIRONMENTAL CLONE 1611 (1)
- [2.23] ENVIRONMENTAL CLONE PAD1 GROUP (3)
- [2.24] ENVIRONMENTAL CLONE PAD39 (1)
- [2.25] FIBROBACTER AND ACIDOBACTERIUM (173)
- [2.26] ENVIRONMENTAL CLONE NH25–19 (1)
- [2.27] SPIROCHETES AND RELATIVES (648)
- [2.28] PROTEOBACTERIA (6893)
- [2.29] FUSOBACTERIA AND RELATIVES (40)
- [2.30] GRAM POSITIVE BACTERIA (4947)

*Division of Proteobacteria* comprises the majority of prokaryotes with Gram-negative type of cell wall (*Gracilicutes* by conventional taxonomy) and includes the following subdivisions by the classification of the Ribosomal Database Project (RDP-II):

- [2.28] PROTEOBACTERIA (6893)
  - [2.28.1] ALPHA\_SUBDIVISION (1968)
  - [2.28.2] BETA SUBDIVISION (1085)
  - [2.28.3] GAMMA SUBDIVISION (2949)
  - [2.28.4] DELTA SUBDIVISION (545)
  - [2.28.5] EPSILON SUBDIVISION (317)
  - [2.28.6] UNCULTURED MAGNETOTACTIC CLONES (27)
  - [2.28.7] ENVIRONMENTAL CLONE\_A8 (1)
  - [2.28.8] UNNAMED DELTA PROTEOBACTERIUM (1)

*Division of Gram-positive bacteria* comprises prokaryotes with Gram-positive negative type of cell wall (*Firmicutes* by conventional taxonomy) and includes the following subdivisions by the classification of the Ribosomal Database Project (RDP-II):

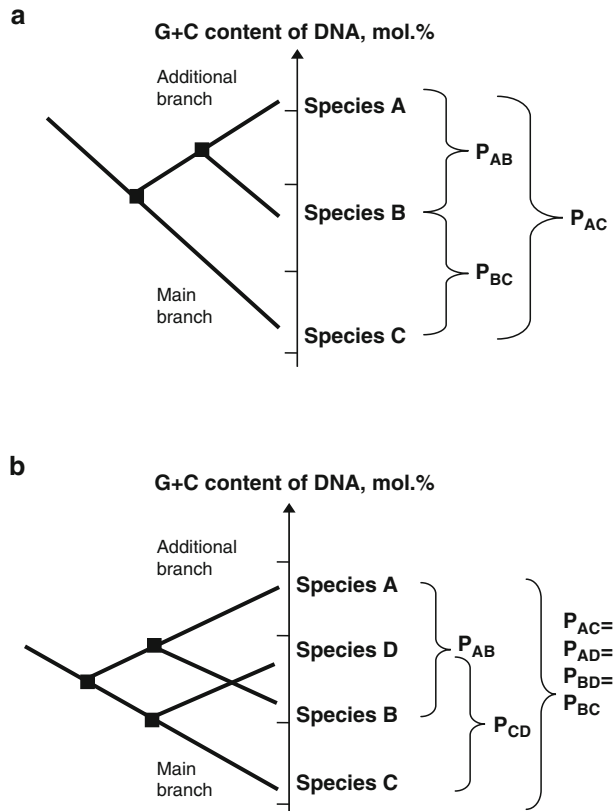
- [2.30] GRAM POSITIVE\_BACTERIA (4947)
  - [2.30.1] HIGH\_G + C\_BACTERIA (2320)
  - [2.30.2] THERMOANAEROBACTER AND RELATIVES (146)
  - [2.30.3] SPOROMUSA AND RELATIVES (133)
  - [2.30.4] EUBACTERIUM AND RELATIVES (299)
  - [2.30.5] C.PURINOLYTICUM\_GROUP (62)
  - [2.30.6] ANAEROBIC\_HALOPHILES (39)
  - [2.30.7] BACILLUS-LACTOBACILLUS-STREPTOCOCCUS\_SUBDIVISION (1248)
  - [2.30.8] MYCOPLASMA AND RELATIVES (377)
  - [2.30.9] CLOSTRIDIUM AND RELATIVES (323)

### 4.3. Connection Between Phylogenetic Grouping and G + C Content of Chromosomal DNA

*G + C content in DNA and phylogenetic grouping.* The G + C content of chromosomal DNA of prokaryotes ranges from 25 to 80 mol%. The current thinking in prokaryotic taxonomy is that if organisms in the same taxon are too dissimilar in G + C content, a taxon should be divided. It is a common opinion in prokaryotic classification that the distributions of G + C content of DNA is important for taxonomy (grouping) but not for phylogenetic classification. The reason for this is an absence of theory connecting the G + C content in DNA with the evolutionary distance between the rRNA sequences.

*A model connecting G + C content in DNA with the rRNA-based phylogenetic distances* includes the following assumptions:

1. A speciation (formation of new species) results in the formation of additional phylogenetic branches on the main branch. Figure 2.5a shows the two modern representatives of main and additional branches.



**Fig. 2.5.** Determination of evolutionary distance (P) between species in terms of G + C content in chromosomal DNA of modern representatives A, B, C, and D on main and additional branches.

2. The G + C contents in the DNA of the representatives in the main and additional evolutionary branches are changed in opposite directions. One branch and its modern representatives can be called GC<sup>+</sup>, while another branch and modern representative can be called GC<sup>-</sup> (Fig. 2.5a). Hypothetically, the bias to GC<sup>+</sup> or GC<sup>-</sup> mode of DNA sequence evolution can be mediated by such conditions of environment as temperature and salinity.
3. The evolutionary distance (in terms of G + C content in chromosomal DNA) between modern representatives of main and additional branches ("GC distance") is the absolute value of the difference between G + C content of modern representatives of these branches (Fig. 2.5a, b).
4. The evolutionary distance (in terms of G + C content in chromosomal DNA) between modern representatives of any two branches (G + C content distance) is the absolute value of the difference in G + C contents in DNA of modern representatives of their main branches (Fig. 2.5a, b).

One proof of this model is that the pattern of the branching of rRNA-based phylogenetic tree for some species corresponds to the G + C content in the DNA of these species. Some demonstrations of this correlation for small numbers of species of methanogens, nitrifiers and sulphate-reducers are shown in Figs. 2.6 and 2.7. Small numbers of species was selected to demonstrate the correlation clearly.

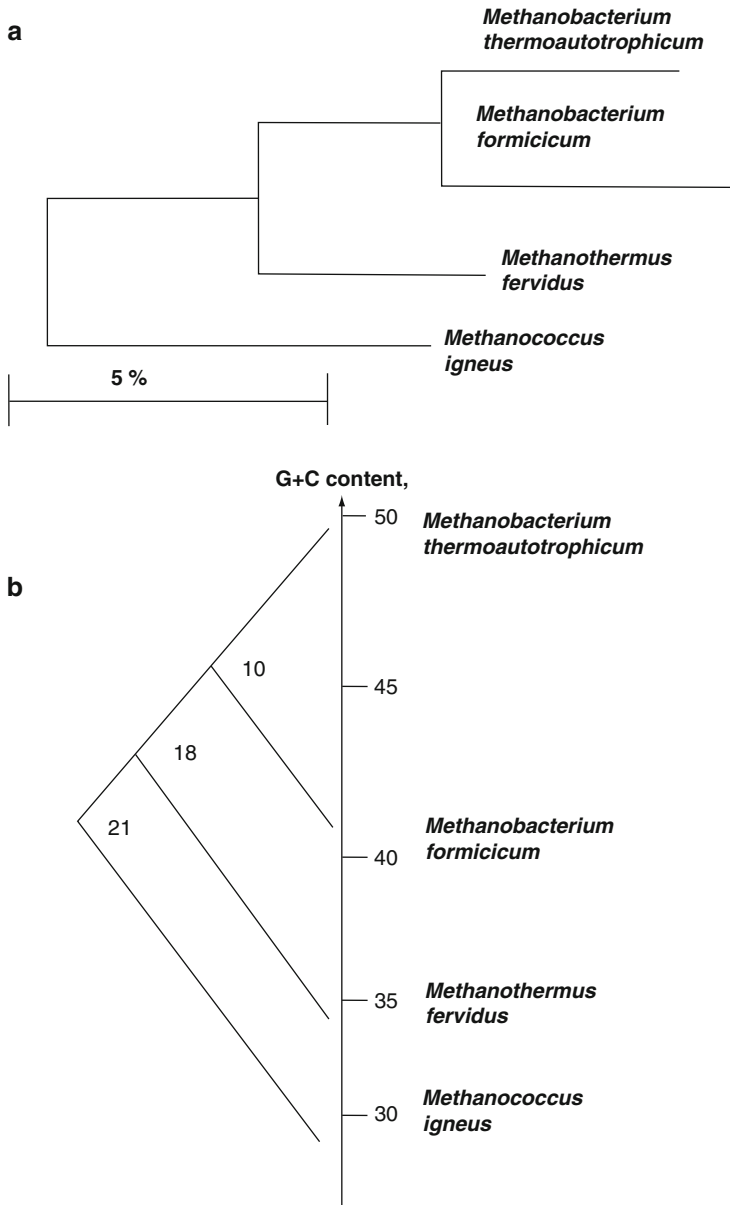
16S rRNA phylogenetic distance trees and the trees based on evolutionary distances in terms of G + C content in DNA show the same order of branching. Therefore, this correlation can be used for the production phylogenetic tree showing not only the evolutionary distances between the sequences of 16S rRNA but also the branching order, which is related to the G + C content of DNA of studied microorganisms. The hypothesis explaining the evolutionary formation of GC<sup>+</sup> and GC<sup>-</sup> branches can be used for the prediction of prokaryotes not yet discovered.

#### ***4.4. Comparison of rRNA-Based Phylogenetic Classification and Conventional Phenotypic Taxonomy***

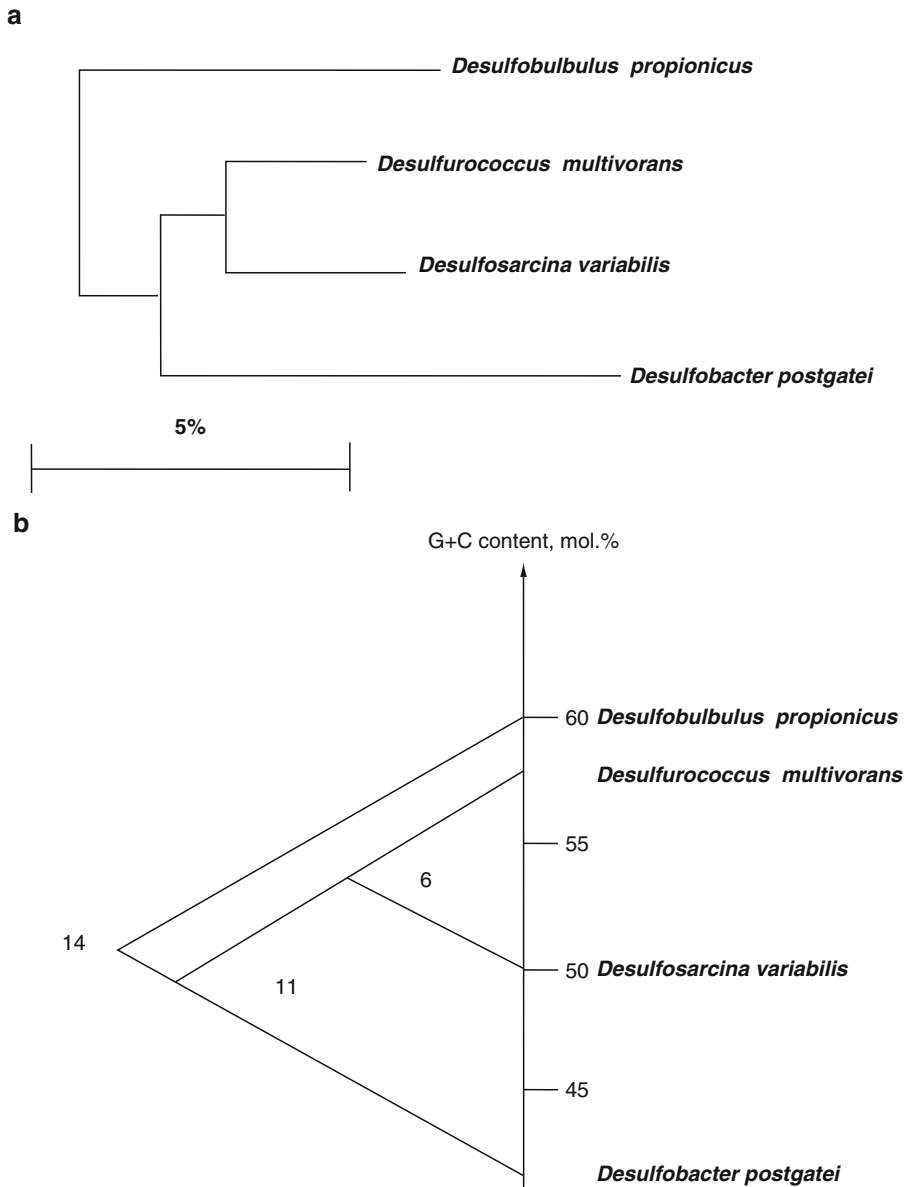
The *main contradiction* between phenotypic taxonomy and modern phylogenetic classification, based on comparison of rRNA sequences is that physiological groups often do not correspond to rRNA-based phylogenetic groups. Some examples are the grouping of microaerophilic and aerobic prokaryotes in one  $\beta$ -subdivision of proteobacteria and grouping of facultative-anaerobic and aerobic prokaryotes in the  $\gamma$ -subdivision of proteobacteria. Almost all divisions and subdivisions consist of species with a mixture of physiological and cytological features.

The small evolutionary distance between two species of different physiological groups reflects a short evolutionary time after speciation. An example is the small evolutionary distance between *Nitrobacter winogradski* and *Rhodospseudomonas palustris*, which are an aerobic chemolithotroph and an anaerobic phototroph, respectively. The small evolutionary distance between their 16S rRNAs can be explained as thus: the branch of *Nitrobacter winogradski*, originated from the line of *Rhodospseudomonas palustris* a short evolutionary time ago.

*Physiological twins and phylogenetic twins.* The contradiction between phenotypic taxonomy and rRNA-based phylogenetic classification is due to the stability of newly formed



**Fig. 2.6.** Demonstration of similarity in the branching of 16S rRNA distance tree (a) and the distance tree accounting G + C content of DNA (b) of some methanogens. Evolutionary distances (P) between the species in terms of G + C content in chromosomal DNA of modern representatives are shown in bear the points of branching.



**Fig. 2.7.** Demonstration of similarity in the branching of 16S rRNA distance tree (**a**) and the distance tree accounting G + C content of DNA (**b**) of some sulphate-reducing bacteria. Evolutionary distances (P) between the species in terms of G + C content in chromosomal DNA of modern representatives are shown in bear the points of branching.

basic physiological properties during evolution. This stability is especially clear for such basic physiological properties as types of energy generation and the relationship of microorganisms to oxygen. At the same time, rRNA and other polynucleotide and polyaminoacid sequences changed significantly during evolution. Therefore, the distance between rRNA sequences, which is used in phylogenetic classification, reflects the time after divergency (branching) of phylogenetic lines but not the physiological discrepancies between their representatives. For example, such basic physiological characteristics as the ability for anaerobic respiration was created at an early stage of evolution and preserved in modern representatives. The physiological properties of some of these representatives may be very similar, but the dissimilarity between their rRNAs would be very large because of the accumulation of a large number of mutations in 16S rRNA over a long period of evolution (Fig. 2.8a).

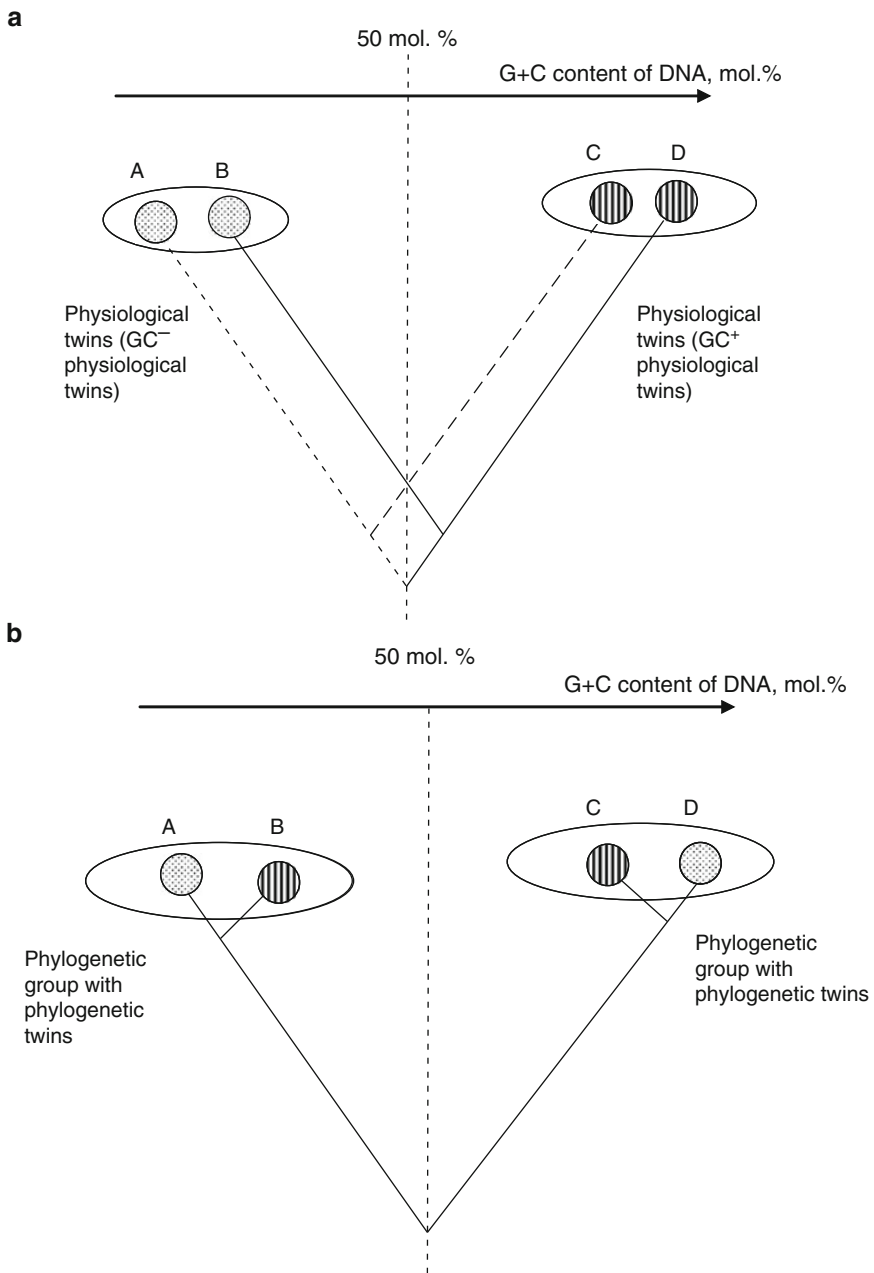
If the main and additional evolutionary branches separated a long time ago but their representatives developed under the same conditions, they can have similar basic physiological features. These representatives, with a large phylogenetic distance, in terms of 16S rRNA sequences and large evolutionary distance, in terms of G + C content in DNA but similar physiological properties, can be considered physiological twins because they belong to the same physiological group (Fig. 2.8a).

If the main and additional evolutionary branches did not separate a long time ago, their modern representatives can have different basic physiological features but a small phylogenetic distance in terms of 16S rRNA sequences and small evolutionary distance in terms of G + C content in DNA (Fig. 2.8b). These organisms, with a small phylogenetic distance in terms of 16S rRNA sequences and a small evolutionary distance in terms of G + C content in DNA but with large physiological differences, can be considered phylogenetic twins because they belong to the same phylogenetic group (Fig. 2.8b).

The existence of the groups of  $GC^+$  and  $GC^-$  physiological twins can be proven by symmetry of the distribution of G + C content in DNA of species within the groups of fermenting, anaerobic respiring and aerobic prokaryotes (Fig. 2.9a, b).

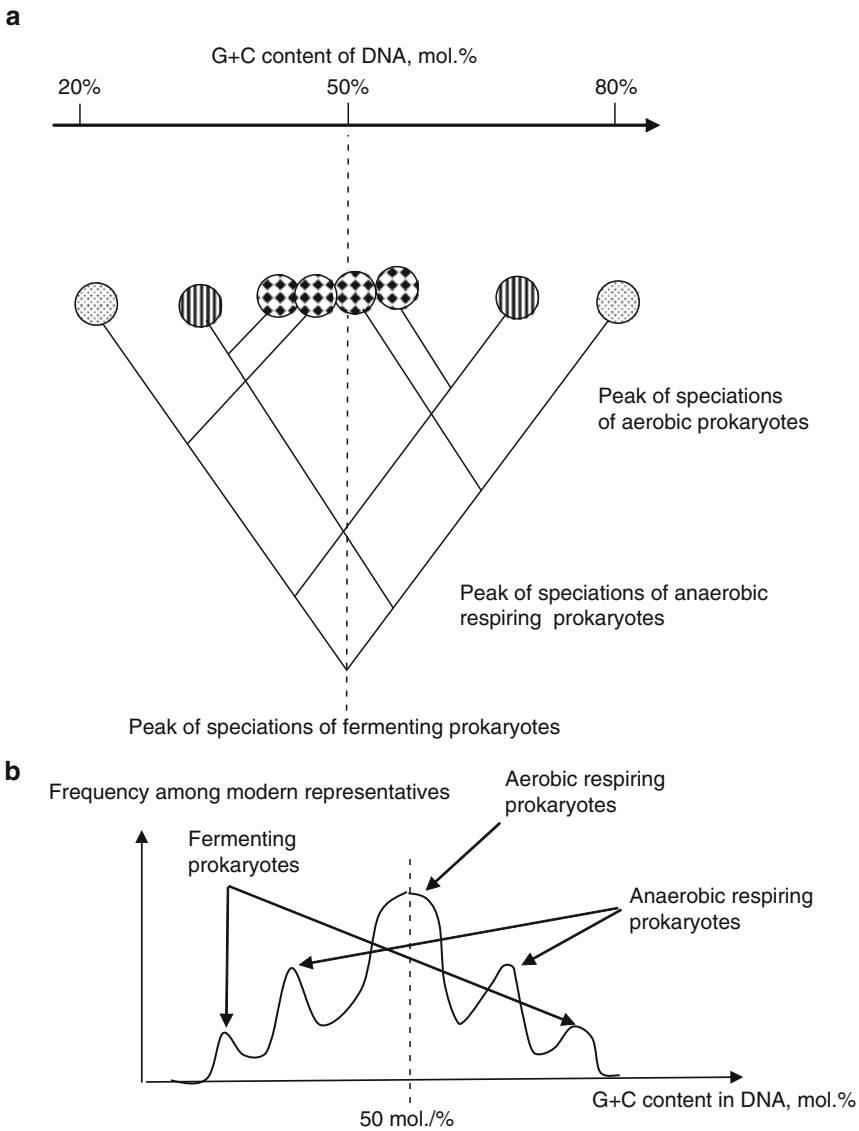
The existence of physiological twins and phylogenetic twins comprises the difference between phenotypic taxonomy and rRNA-based phylogenetic classification. Another cause of this difference is that the most popular present phylogenetic grouping is based on the evolution of one gene of 16S rRNA. Evolutionary classification based on the group of genes may be different, especially if these genes will be related to energy generation and oxidation–reduction reactions. However, even for such complicated phylogenetic analysis, the reason for the basic contradiction between phylogenetic and physiological groupings will remain: physiological and cytological features acquired in evolution remain basically the same, but the gene sequences will be significantly changed during evolution.

Identification of prokaryotes by the sequencing of 16S rRNA gene is faster and simpler than identification based on physiological features. Additionally, the sequences from poorly cultivated prokaryotic strains, or those not cultivated in the laboratory, can also be used for the identification of prokaryotes. That is why phylogenetic classification, based on comparison of 16S rRNA gene, is so popular at present. However, due to the contradictions between phylogenetic classification and physiological grouping, the mechanistic classification of prokaryotes, using only similarities of 16S rRNA gene produces the groups, which are



**Fig. 2.8.** Formation of physiological twins (a) and phylogenetic twins (b).





**Fig. 2.9.** Waves of speciations (a) and symmetry of the distribution of G + C content in DNA in the groups of fermenting, anaerobic respiring, and aerobic prokaryotes (b).

often the mixture of species with different basic physiological properties. Thus, to classify prokaryotes without contradictions between physiological and phylogenetical grouping, data on the evolution of basic physiological properties and DNA sequences must be combined and classified altogether.

*Parallelism in evolution of genes.* Another source of difference between phenotypic taxonomy and rRNA-based phylogenetic classification is the parallelism in the evolution of genes. In rRNA-based phylogenetic classification, it is thought that the number of differences in sequence of rRNA reflects the evolutionary distance of the origin of compared sequences from a common ancestor sequence. However, it was proved in experiments that there may be lateral transfers of genes in the environment, i.e., transfer of genes not only from ancestor to descendant but also between neighboring organisms. By our hypothesis of parallelism of prokaryotic evolution described below, the frequency of lateral transfer of the genes between major phylogenetic lines of *Gracilicutes*, *Firmicutes* and *Archaea* can be synchronized by the evolutionary changes of atmosphere and the waves of organic matter accumulation due to the waves of glaciations on the planet.

#### 4.5. Periodic Table of Prokaryotes

*Absence of predictive power in rRNA-based phylogenetic classification.* The existing classification of 16S rRNA gene shown in the Ribosomal Database Project (RDP-II) and other databases, for example, BLAST of National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/>), is very useful for the experimental identification of microbial species. Identification can be currently performed by PCR of 16S rRNA gene and gene sequencing for several hours. Using conventional taxonomy methods to identify species requires a significantly longer and more laborious procedure performed by experienced researchers. Therefore, rRNA-based classification is more popular in experimental research. However, there are no physiological connections between phylogenetic groups and no predictive power in the current rRNA-based phylogenetic classification. As a result of the widespread use of 16S rRNA-based phylogenetic classification in experimental research, microbial diversity is often perceived by new users and students as a random mixture of microbial species and groups.

*Main features of periodic table of prokaryotes.* The hypothetical periodic table of prokaryotic phylogeny (Table. 2.3) was proposed to give predictive power to prokaryotic classification, clarify the physiological and evolutionary connections between microbial groups and give a logical basis for students to understand microbial diversity. (8, 11).

The main features of the periodic table of prokaryotic phylogeny are described below.

- A. Three basic phylogenetic lines (related to the divisions of Bergey's Manual of Systematic Bacteriology) underwent parallel synchronized evolution:
  1. L1, *Gracilicutes* (prokaryotes with thin cell walls, implying a Gram-negative type cell wall).
  2. L2, *Firmicutes* (prokaryotes with a thick, strong skin, indicating a Gram-positive type cell wall).
  3. L3, *Mendosicutes* (Archaea, prokaryotes that lack a conventional peptidoglycan).

The divisions of *Tenericutes* from Bergey's Manual (prokaryotes without rigid cell wall) and oligotrophs (prokaryotes adapted for growth at a low concentration of carbon source in a medium) are not considered basic phylogenetic lines because they both do not have chemo- and phototrophic modern representatives with all types of energy generation.

**Table 2.3**  
**Phylogenetic lines, periods, and groups of periodic table of prokaryotes (selected examples of conventional genera are shown in the groups)**

Evolutionary line of prokaryotes	Sub-line	Periods of evolution			
		P1	P2	P3	P4
Prokaryotes with Gram-negative type of cell wall ( <i>Gracilicutes</i> )	L1c	P1 – L1c <i>Bacteroides</i> <i>Prevotella</i> <i>Ruminobacter</i>	P2-L1c <i>Desulfobacter</i> <i>Geobacter</i> <i>Wolinella</i>	P3-L1c <i>Escherichia</i> <i>Shewanella</i> <i>Beggiatoa</i>	P4-L1c <i>Pseudomonas</i> <i>Acinetobacter</i> <i>Nitrosomonas</i>
	L1p	P1-L2p Not known	P2-L1p <i>Chlorobium</i> <i>Rhodocyclus</i> <i>Chromatium</i>	P3-L1p <i>Chloroflexus</i>	P4-L1p cyanobacteria <i>Prochloron</i>
Prokaryotes with Gram-positive type of cell wall ( <i>Firmicutes</i> )	L2c	P1-L2c <i>Clostridium</i> , <i>Peptococcus</i> <i>Eubacterium</i>	P2-L2c <i>Desulfotomaculum</i> <i>Desulfitobacterium</i> <i>Bacillus infernus</i>	P3-L2c <i>Microthrix</i> , <i>Nocardia</i> , <i>Streptococcus</i>	P4-L2c <i>Bacillus</i> <i>Arthrobacter</i> <i>Streptomyces</i>
	L2p	P1-L2p <i>Heliobacterium</i> <i>Heliobacillus</i>	P2-L2p Not known	P3-L2p Not known	P4-L2p Not known
<i>Archaea</i>	L3c	P1-L3c <i>Desulfurococcus</i> <i>Thermosphaera</i> <i>Pyrodictium</i>	P2-L3c <i>Methanobacterium</i> <i>Thermococcus</i> <i>Haloarcula</i>	P3-L3c <i>Metallosphaera</i> <i>Sulfolobus</i> <i>Acidianus</i>	P4-L3c <i>Picrophilus</i> <i>Ferroplasma</i>

B. Phototrophic and chemotrophic sublines underwent parallel synchronized evolution in every phylogenetic line, with the exemption of the phototrophic line of *Archaea*. Therefore, there are five phylogenetic sublines in the periodic table:

1. L1c, chemotrophic gracilicutes.
2. L1p, phototrophic gracilicutes.
3. L2c, chemotrophic firmicutes.
4. L2p, phototrophic firmicutes.
5. L3c, chemotrophic archaea.

Some representatives of *Archaea* possess light energy assimilating pigments but there are no phototrophic representatives in this line, probably because of the extreme environmental conditions for which *Archaea* were adapted.

C. New chemotrophic ways of biologically available energy production were created in every chemotrophic subline and related period of evolution in the following sequence:

1. P1c, anaerobic fermentation.
2. P2c, anaerobic respiration.
3. P3c, microaerophilic respiration or alteration of fermentation and respiration.
4. P4c, aerobic respiration.

- D. New phototrophic ways of biologically available energy production were created in every phototrophic subline and related period of evolution in the following sequence:
1. In the period P1p, products of anaerobic fermentation (organic acids and alcohols) are used as electron donor and carbon sources.
  2. In period P2p, products of anaerobic respiration are used as electron donors ( $\text{H}_2\text{S}$ ,  $\text{Fe}^{2+}$ ) and carbon sources ( $\text{CO}_2$ ).
  3. In period P3p, products of microaerophilic respiration are used as electron donors (S) and carbon sources ( $\text{CO}_2$ ).
  4. In period P4p, products of aerobic respiration are used as electron donors ( $\text{H}_2\text{O}$ ) and carbon sources ( $\text{CO}_2$ ).

*Reasons of parallelism and periods in periodic table of prokaryotes.* The existence of three evolutionary lines can be explained by parallel synchronized evolution in three different habitats:

1. *Gracilicutes* (Gram-negative bacteria) were adapted to life in biotops with constant osmotic pressure such as seawater or animal fluids.
2. *Firmicutes* (Gram-positive bacteria) were adapted to life in biotops with changeable osmotic pressure such as soil and shallow basins.
3. *Archaea* were adapted to life in hyper-extreme environments with high temperature and salinity.

The evolutionary parallelism in phylogenetic lines can be explained by the geological synchronization of the frequency of speciations in three phylogenetic lines. Hypothetically, the synchronization could have been caused by the waves of glaciations in the Pre-Cambrian era (8, 11). Every period of glaciation decreased the microbial population in the biosphere, decreased the concentration of  $\text{CO}_2$  in the atmosphere and increased the accumulation of dead organic matter, thus creating conditions for a new wave of speciations in a warm period of geological evolution on Earth.

Another factor in the synchronization of the evolutionary periods in phylogenetic lines was the evolution of the anaerobic atmosphere to an aerobic one. Therefore, the periods of prokaryotic evolution and related groups of anaerobic fermenting, anaerobic respiring, microaerophilic and aerobic prokaryotes could be synchronized by the changes of oxygen concentration in the atmosphere. The creation of new groups of chemotrophs likely produced the conditions for the creation of a new group of phototrophs because the final products of this new group were used as electron acceptors and carbon sources for a related new group of phototrophs. Also likely, the horizontal gene transfers between three habitats enhanced the synchronization of parallel evolution in three phylogenetic lines.

*Importance of the periodic table of prokaryotic phylogeny.* The periodic table of prokaryotic phylogeny, shown in Table. 2.3, provides a theoretical understanding of microbial diversity. Due to the logical connection between the Earth's evolution and the parallel evolution in three lines of prokaryotes, the table possesses predictive power. Some groups of prokaryotes have not been discovered yet. Using the periodic table of prokaryotic phylogeny, it would be possible to forecast the discovery of aerobic and microaerophilic phototrophic *Firmicutes* (Gram-positive bacteria).

The periodic table of prokaryotic evolution gives a general overview and is not suitable for practical taxonomy and identification. However, it would be possible to produce more detailed identification tables, taking into account the parallelism of not only physiological

but additional cytological, biochemical, ecological and molecular-biological features of prokaryotes. To avoid the contradictions between the classification by the periodic table and phylogenetic classification, based on 16S rRNA gene sequences, the existence of physiological twins and phylogenetic twins described above must be taken into account.

## 5. FUNCTIONS OF MICROBIAL GROUPS IN ENVIRONMENTAL ENGINEERING SYSTEMS

### 5.1. Functions of Anaerobic Prokaryotes

These microorganisms are related to the period P1 of the periodic table. Their natural biotops are the sediments of aquatic ecosystems, tissues of macroorganisms, anaerobic micro-zones of soil and hot springs.

*Chemotrophic subline of Gracilicutes (group P1-L1c)*. The main functions of anaerobic chemotrophic gram-negative fermenting bacteria (group P1-L1c) in environmental biotechnology are as follows:

1. Fermentation of saccharides to organic acids, alcohols and hydrogen.
2. Syntrophic formation of acetate from other organic acids during anaerobic digestion of organic matter.
3. Indication of fecal pollution of water.

Examples of the functions of selected genera are shown below.

*Bacteroides spp.* are the predominant organisms in the human colon and are generally isolated from the gastrointestinal tract of humans and animals; some species are pathogenic. Their functions in environmental biotechnology include: (a) anaerobic degradation of polysaccharides in an anaerobic digester and the anaerobic zones of microbial biofilms; and (b) indication of fecal pollution in water. *Prevotella spp.* are mainly pathogenic organisms but some species can be used for the biodegradation of collagen-containing wastes. *Ruminobacter spp.* facilitate anaerobic fermentation in the rumen and can be used for anaerobic fermentation of organic wastes. *Acetogenium spp.* and *Syntrophococcus spp.* are so-called acetogens, capable of producing acetate from other organic acids. This function ensures a supply of acetate to acetotrophic methanogens in the anaerobic digestion of organic wastes. *Syntrophococcus spp.* can also metabolize some C<sub>1</sub>-compounds (methanol, formaldehyde, formic acid, carbon monoxide) and remove methoxyl groups from lignin. *Syntrophobacter wolinii* also produces acetate by degradation of propionate only in coculture (syntrophically) with hydrogen-utilizing prokaryotes. The acetate produced is sequentially used by acetotrophic methanogens during anaerobic digestion of organic wastes. *Veillonella spp.* are parasitic microorganisms. Some species can be used for the reduction of nitroaromatic compounds, including 2,4,6-trinitrotoluene (TNT), as first step in their biodegradation.

*Chemotrophic subline of Firmicutes (group P1-L2c)*. The main functions of anaerobic chemotrophic gram-positive fermenting bacteria (group P1-L2c) in environmental biotechnology are as follows:

1. Hydrolysis of biopolymers.
2. Fermentation of saccharides and aminoacids to organic acids, alcohols and hydrogen.

3. Formation of acetate from hydrogen and carbon dioxide during the anaerobic digestion of organic matter.
4. Indication of fecal pollution in water.

Examples of the functions of selected genera are shown below. Anaerobic chemotrophic Gram-positive fermenting bacteria (group P1-L2c) perform the stages of anaerobic digestion of organic wastes such as the hydrolysis of biopolymers and fermentation of monomers to organic acids, alcohols, hydrogen and carbon dioxide. The existence of GC antipodes is especially clear in this group. This group is subdivided in both phylogenetic classification and conventional taxonomy for the divisions with low and high G + C content of DNA. Examples of the functions of selected genera from the subdivision with high GC content of DNA are shown below.

Species from the genus *Clostridium* are able to form endospores; usually they have no tolerance towards oxygen but the spores can survive in an aerobic environment. Both pathogenic and nonpathogenic species exist. The pathogenic species, for example, *Clostridium botulinum* and *Clostridium tetani*, are agents of severe diseases and produce strong toxins that can be considered as bioweapons. Clostridia can hydrolyze biopolymers, ferment monomers and aminoacids, produce alcohols, organic acids and hydrogen. It is the first rate-determining step in the anaerobic digestion of organic waste. Some thermophilic clostridia can be used for the hydrolysis of cellulose and production of fuel ethanol. Some clostridial species may be used for the reductive dechlorination of xenobiotics, for example, pesticides and herbicides. *Clostridium perfringens* is an indicator species in water quality evaluation. Spores of *Clostridium spp.* are used as test cultures in disinfection studies.

Species from the genera *Clostridium*, *Peptococcus*, *Peptostreptococcus* and *Eubacterium* ferment saccharides to form fatty acids (butyric, propionic, lactic, succinic and acetic acids), ethanol, hydrogen and carbon dioxide. It is the second step in the anaerobic digestion of organic waste.

Species from genera *Acetobacterium* and *Acetogenium*, together with some species from the genus *Clostridium*, are homacetogenic bacteria; they are able to reduce carbon dioxide and produce acetate that serves as a substrate for acetotrophic methanogens. It is the third important step in the anaerobic digestion of organic wastes. The production of organic acids by anaerobic fermenting bacteria can contribute to microbially induced corrosion of metal engineering systems.

*Chemotrophic subline of Archaea (group P1-L3c)*. There are known species of anaerobic fermenting *Archaea*. Obligate anaerobic species from genera *Desulfurococcus*, *Thermosphaera*, *Pyrodictium* and some others from the phylum *Creanarchaeota* are able to ferment organic substances, but some of them can generate energy using sulphur as an electron acceptor. Those species capable of sulphur respiration can be classified also in the group of anaerobic *Archaea* (group P2-L3c of periodic table). These species are not currently used in environmental engineering systems but they may be potentially effective in the anaerobic biodegradation of organic wastes in engineered hot ecosystems.

*Phototrophic subline of Gracilicutes (group P1-L1p)*. In the future, such organisms may be found among filamentous anoxygenic phototrophs (FAPs), belonging to the family *Chloroflex-*

*aceae*. Anaerobic gram-negative phototrophic bacteria using oxidized products of fermentation (organic acids and alcohols) can perform anaerobic photobiodegradation of organics in stabilization ponds and microbial mats of shallow rivers or springs.

*Phototrophic subline of Firmicutes (group P1-L2p)*. There is one family *Heliobacteriaceae* in this group of obligate anaerobic gram-positive phototrophic bacteria. The genera of heliobacteria are *Heliobacterium*, *Heliobacillus*, *Heliophilum* and *Heliorestis*. Heliobacteria catabolize mainly fermentation products such as pyruvate, lactate, acetate, butyrate, ethanol and carbon dioxide. They can perform fermentation of pyruvate with the formation of acetate and CO<sub>2</sub> as products. Heliobacteria reside in soil, especially in paddy fields, mainly in the rhizosphere of plants, and can form spores for survival under unfavourable growth conditions. Symbiotic relationships may exist between heliobacteria performing strong nitrogen fixation for rice plants and rice plants supplying organic substances for heliobacteria. Heliobacteria would be useful in the biodegradation of organics in soil or microbial mats of springs, and in nitrogen fixation in paddy soils and soils under bioremediation.

## 5.2. Functions of Anaerobic Respiring Prokaryotes

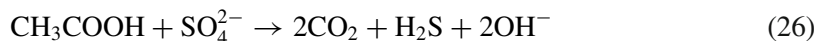
These microorganisms are related to the period P2 of the periodic table. Their natural biotops are anoxic zones of aquifers, aquatic sediments, hot springs and anoxic microzones of soil.

*Chemotrophic subline of Gracilicutes (group P2-L1c)*. There are many important functions of anaerobic chemotrophic gram-negative anaerobic respiring bacteria (group P2-L2c) in environmental biotechnology:

1. Biotechnologies, coupled with the reduction of sulphate: removal of heavy metals or sulphate.
2. Biotechnologies, coupled with the reduction of nitrate and nitrite: denitrification of wastewater; anoxic biodegradation of organic substances.
3. Biotechnologies coupled with the reduction of Fe<sup>3+</sup>: removal of phosphate, anoxic biodegradation of organic substances.

Examples of the functions of selected genera are shown below.

Dissimilatory sulphate-reducing bacteria are obligate anaerobes that use organic acids, alcohols and hydrogen as donor of electrons and sulphate or other oxoanions of sulphur as acceptors of electrons, for example:

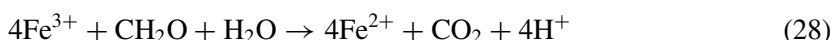


Hydrogen sulphide is the toxic product of this anaerobic respiration. There is a large diversity of morphological forms (spherical, ovoid, rod-shaped, spiral, vibrioid-shaped cells, etc.), physiological varieties and related genera in phenotypic taxonomy, for example, *Desulfococcus*, *Desulfobacter*, *Desulfobacterium*, *Desulfobulbus*, *Desulfosarcina*, *Desulfovibrio*, etc. Sulphur-reducing bacteria from genera *Desulfurella* and *Desulfuromonas* are unable to reduce sulphate or other oxoanions of sulphur. The groups of sulphate-reducing and sulphur-reducing bacteria are concentrated in a delta subdivision of Proteobacteria in phylogenetic classifications. Typical habitats are anoxic sediments or bottom waters of freshwater, marine

or hypersaline aquatic environments; thermophilic species occur in hot springs and submarine hydrothermal vents. Sulphate-reducing bacteria are the agents of corrosion of steel and concrete constructions, and engineering equipment in oil and gas industry and in wastewater treatment. These bacteria can be used in environmental engineering to precipitate undissolved sulphides of heavy metals from solutions or for the removal of sulphate from wastewater with a high concentration of sulphate.

Close phylogenetic relatives of sulphate-reducing bacteria in a delta subdivision include a group of genus, *Syntrophus*, benzoate-reducing bacteria, oxidizing fatty acids with benzoate as an electron acceptor in syntrophic association with hydrogen-using microorganisms such as methanogen *Methanospirillum hungatei*.

Iron-reducing bacteria can reduce different Fe(III) compounds, using organic substances. These bacteria are important in the anaerobic biodegradation of organic matter in the aquifers because they can reduce different Fe(III) natural compounds such as iron-containing clay minerals:



This group of bacteria includes many genera such as *Geobacter*. Species of genus *Geobacter* are from a delta subdivision of Proteobacteria in the 16S rRNA-based phylogenetic classification. They are phylogenetically similar to sulphate-reducing bacteria and compete with them for electron donors in anaerobic zones. *Geobacter metallireducens* and *Geobacter sulfurreducens* are able to reduce not only Fe(III) but also Mn (VI), U(VI), Tc(VII), Co(III), Cr(IV), Au(III), Hg(II), As(V) and Se(VII), using aliphatic and some aromatic acids and alcohols as electron donors. It is the dominant group of iron-reducing bacteria detected in aquifers and subsurface environments. Therefore, they can be used for the bioremediation of these biotops. Other biotechnologies involving iron-reducing bacteria, are the removal of phosphate, sulphide, and ammonia from return liquor of municipal wastewater treatment plants (12, 13). Two new cultures of iron-reducing bacteria, *Stenotrophomonas maltophilia* and *Brachymonas denitrificans*, have been recently isolated. These cultures were able to remove phosphate and degrade xenobiotics, using iron hydroxide as an oxidant and branched fatty acids of liquid after anaerobic digestion of biomass (13).

Halobacteria from *Dehalobacter* genus are capable to oxidize such electron donors as formate, acetate, pyruvate, lactate and H<sub>2</sub> due to anaerobic reductive dechlorination and can be used for degradation of chlorinated ethenes in soil or wastewater. The ability to reduce Fe(III), Mn (VI), Se (VI) and As (V) and anaerobic reductive dechlorination is often a common property amongst bacteria in the group P2-L1.

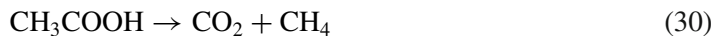
The species *Wolinella succinogenes* can use fumarate, nitrate, nitrite, nitrous oxide (N<sub>2</sub>O) and polysulphide as terminal electron acceptors with formate, molecular hydrogen or sulphide as the electron donors. The species was proven as a bioagent for the treatment of hazardous industrial wastewater containing ammonium perchlorate (AP) and rocket motor components on the sites of demilitarization and disposal of solid rocket motors.

Denitrifying bacteria, which are capable of oxidizing organic substances, hydrogen, Fe<sup>3+</sup>, H<sub>2</sub>S or S using nitrite or nitrate as electron acceptors, are usually not only anaerobic respiring bacteria but facultative anaerobic prokaryotes from the groups P3-L1 and P3-L2.



*Chemotrophic subline of Firmicutes (group P2-L2c).* The functions of anaerobic chemotrophic Gram-positive anaerobic respiring bacteria (group P2-L2c) in environmental biotechnology are similar to the functions of the bacteria from P2-L1c group, i.e., biotechnologies, coupled with reduction of sulphate, nitrate, Fe(III) and other metals. Sulphate-reducing bacteria in this group are classified in the genus *Desulfotomaculum*. They form heat-resistant endospores and use organic acids and alcohols as electron donors. The *Desulfitobacterium* genus was recognized as an important group of anaerobic dechlorination of such xenobiotics as chlorinated phenols, chlorinated ethenes and polychlorinated biphenyls (PCBs). The species of *Desulfosporosinus* genus may be important in the bioremediation of groundwater, contaminated with benzene, toluene ethylbenzene and xylene (BTEX compounds). *Bacillus infernus* is an anaerobic species that is able to reduce Fe(III) and Mn (VI), using formate or lactate.

*Chemotrophic subline of Archaea (group P2-L3c).* The majority of *Archaea*, the methanogens and extreme (hyper) thermophiles are representatives of this group. Methanogens are obligate anaerobes, which convert CO<sub>2</sub>, molecular hydrogen, methyl compounds or acetate to methane by anaerobic respiration:



Methane is produced by methanogens in the rumen (fore-stomach) of ruminant animals, paddy fields and wetlands. It is also produced and utilized as a fuel during industrial anaerobic digestion of organic wastes on municipal wastewater treatment plants and on the landfills. Representatives of microbial genera *Methanobacterium*, *Methanobrevibacter*, *Methanothermobacter*, *Methanococcus*, *Methanobolbus*, *Methanotherrix*, *Methanomicrobium*, *Methanogenium*, *Methanospirillum*, *Methanoplanus*, *Methanocorpusculum*, *Methanoculleus*, *Methanohalobium*, *Methanohalophilus*, *Methanosarcina* and *Methanosphaera* can be easily distinguished under a TEM by the shape of cell or cell arrangement or under CLSM, using specific oligonucleotide probes and FISH with cells of methanogens.

Sulphate-reducers (*Archaeoglobus*) and extremely thermophilic and hyperthermophilic S<sup>0</sup> – metabolizers of *Archaea* (*Desulfurolobus*, *Metallosphaera*, *Pyrobaculum*, *Thermofilum*, *Thermoproteus*, *Hyperthermus*, *Staphylothermus*, *Thermodiscus*, *Desulfurococcus*, *Pyrodictium*, *Thermococcus* and *Pyrococcus*) require temperatures from 70 to 105°C for growth. Some organisms use sulphur as an electron acceptor. Hyperthermophiles are inhabitants of hot and sulphur-rich volcanic springs on the surface or on the ocean floor. They are not used in environmental biotechnology currently, but they may be useful in thermophilic biodegradation of organic wastes, production of environmentally useful enzymes, recovery of metals at a temperature close to boiling water (14), and probably, for the removal of sulphur from coal and oil.

*Phototrophic subline of Gracilicutes (group P2-L1p).* The majority of anoxygenic phototrophic bacteria are in this group. Their main functions in environmental biotechnology are as follows:

1. Anaerobic photoremoval of bad-smelling and toxic H<sub>2</sub>S during anaerobic treatment of waste.

2. Removal of sulphate from water by a bacterial system consisting of sulphate-reducing bacteria and anoxygenic phototrophic bacteria with the formation of elemental sulphur.
3. Anaerobic removal of nutrients.
4. Removal of  $\text{Fe}^{2+}$  from water by photooxidation.

The stoichiometry of anoxygenic photosynthesis can be shown by the following equation:



where  $< \text{CH}_2\text{O} >$  is a conventional formula, showing synthesized carbohydrates.

The following are groups of anoxygenic phototrophic Gram-negative bacteria by conventional taxonomy:

1. Purple sulphur bacteria with internal or external sulphur granules, e.g., the genus *Chromatium*; by the phylogenetic classification of 16S rRNAs, phototrophic purple bacteria belong to the  $\alpha$ -,  $\beta$ - and  $\gamma$ -Proteobacteria.
2. Purple nonsulphur bacteria, e.g., the *Rhodocyclus* genus; according to the phylogenetic classification of 16S rRNAs, phototrophic purple nonsulphur bacteria can be found in the  $\alpha$ - and  $\beta$ -Proteobacteria.
3. Green sulphur bacteria, e.g., the genus *Chlorobium*; by the phylogenetic classification of 16S rRNAs phototrophic green sulphur bacteria can be found in the group 2.16 of bacteria; they are typical aquatic microorganisms and grow where light reaches the anaerobic water layer of a lake or sediment.
4. Multicellular filamentous green nonsulphur bacteria, e.g., the genus *Oscillochloris*, live in hot and cold springs, freshwater lakes, river water and sediments, in both marine and hypersaline environments. It is the group 2.7 of bacteria by the phylogenetic classification of 16S rRNAs and is phylogenetically distant from green sulphur bacteria.

Some green no-sulphur bacteria, such as the species of genus *Chloroflexus*, are able to perform aerobic respiration and may be included in the groups of microaerophilic or aerobic phototrophs. Some species of purple bacteria, green sulphur bacteria and *Chloroflexus* oxidize ferrous iron as an electron donor for photosynthesis instead of  $\text{H}_2\text{S}$ .

*Phototrophic subline of Firmicutes (group P2-L2p)*. Representatives of this group of anoxygenic Gram-positive phototrophic bacteria, using products of anaerobic respiration ( $\text{H}_2\text{S}$ ,  $\text{Fe}^{2+}$ ) have not been discovered yet. Such species will likely have properties close to heliobacteria and will be similar to 16S rRNA phylogeny to the subgroups of heliobacteria, *Desulfotomaculum* and *Desulfitobacterium*.

### 5.3. Functions of Facultative Anaerobic and Microaerophilic Prokaryotes

It is probable that the microorganisms created in period P3, at the boundary of the periods of anaerobic and aerobic atmosphere on Earth, selected two strategies of adaptation:

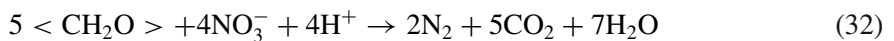
1. The ability to switch methods of energy production between fermentation, anaerobic and aerobic respirations, depending on redox conditions (concentration of oxygen) in their habitat; this ability is characteristic of diverse groups of facultative anaerobic (another term is facultative aerobic) microorganisms; modern representatives of this group dominate in biotops where aerobic and anaerobic conditions frequently change.

- The ability for aerobic respiration only at an oxygen concentration lower than 1 mg/L; this physiological group consists of microaerophilic prokaryotes; the natural habit of modern representatives of this group is the interphase between aerobic and anaerobic zones of the ecosystem or hot aquatic biotops, where the concentration of dissolved oxygen is low.

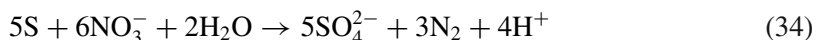
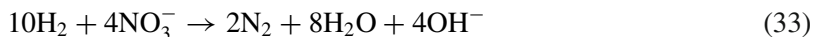
*Chemotrophic subline of Gracilicutes (group P3-L1c)*. Facultatively, aerobic Gram-negative bacteria can produce energy by aerobic respiration, anaerobic respiration (usually by denitrification) or fermentation. Most of these bacteria are active destructors of organic substances and are used in environmental biotechnology where aerobic and anaerobic conditions are frequently changed. Many facultative anaerobic species are enterobacteria, i.e., their typical habitat is the human or animal intestine. There are many agents of water-borne diseases in the genera of *Salmonella*, *Shigella* and *Vibrio*. The cell number of the indicator species of *Escherichia coli*, group of physiologically similar coliforms, and enterococci, are common indicators of water pollution by feces or sewage.

The species of the genus *Shewanella* from the gamma-subdivision of Proteobacteria are facultative anaerobic bacteria, which are able to perform anaerobic respiration using thiosulfate, elemental sulfur, nitrate, iron (III), and manganese (VI) as electron acceptors. The growth yield of these processes is low. However, the biomass of these species can be grown aerobically with high yield on fermentation end products, i.e., lactate, formate and some amino acids. *Shewanella spp.* live naturally in association with fermentative prokaryotes that supply them the needed nutrients. Some species have been isolated from the deep sites and are tolerant of high pressures and low temperatures. The main application of *Shewanella spp.* in environmental biotechnology may be the aerobic growth of biomass with a further application for anoxic remediation of polluted sites, using Fe(III) of ferric oxides, hydroxides, or iron-containing clay minerals as electron acceptors. Reduction of iron and manganese makes these metals dissolved but reduction of dissolved U(VI) and Cr(VI) makes U(IV) and Cr(III) insoluble, respectively.

Denitrifying bacteria are capable of oxidizing organic substances, hydrogen,  $\text{Fe}^{3+}$ ,  $\text{H}_2\text{S}$  or S, using nitrite or nitrate as electron acceptors:



The ability to reduce nitrate or nitrite is widespread among prokaryotes of the P2 and P3 periods. Active denitrifiers, which are used for the removal of nitrate from groundwater and wastewater, are *Pseudomonas denitrificans* and *Paracoccus denitrificans*. Electron donors for industrial scale denitrification can be methanol or ethanol. Hydrogen or sulphur can be used for industrial scale autotrophic denitrification of drinking water and seawater, respectively:



Denitrifiers can also be used for the anoxic biodegradation of toxic organic substances in the case of fast bioremediation of anoxic clay soil.

Filamentous microaerophilic  $\text{H}_2\text{S}$ -oxidizing bacteria from genera *Beggiatoa* and *Thiothrix*, cause a problem of wastewater treatment called bulking. Bulking or bulking foaming in poorly

aerated or overloaded aerobic tanks may be because of the excessive growth of filamentous bacteria forming loose and poorly settled flocs. Growth of *Beggiatoa spp.* in sulphide rich, microaerophilic environment leads to the formation of sulphur-containing slime. Similar hydrogen and sulphide-oxidizing thermophilic filamentous microaerophilic bacteria include species from *Aquifex* and *Hydrogenobacter* genera.

Neutrophilic iron-oxidizing and manganese-oxidizing bacteria are used in environmental biotechnology for the removal of iron and manganese from water. These were also proposed to be used for the removal or even recovery of ammonia from wastewater instead of nitrification (12). Iron-oxidizing bacteria clog drains, pipes and wells with iron hydroxide deposits. Their natural habitats are springs from iron-rich soil, rocks and swamps. Species from *Siderocapsa* genus are usually suspended or attached to the soil, rock and plant surfaces in springs or lakes with an input of Fe(II) from iron-rich soil, deposits or swamps. Cells are covered by a slime iron hydroxide-containing capsule. Species from *Naumanniella* genus are usually psychrophilic and attached to the walls of the pipes and wells. Cells are slim rods with a thin iron-containing capsule. *Siderococcus spp.* are cocci with capsule-like iron hydroxide deposition. *Siderocystis spp.* form chain-connected spherical ferric hydroxide particles. There are also stalk-forming bacteria and thread-forming sheathed bacteria of *Gallionella*, *Leptothrix* and *Sphaerotilus* genera capable of oxidizing iron (II) and precipitate iron (III) hydroxides in the stalk or sheath. Sheaths of neutrophilic iron-oxidizing bacteria can adsorb heavy metals and radionuclides from hazardous streams. The microaerophilic filamentous species of *Sphaerotilus natans* have false branches of sheathed cells with a mycelium-like appearance and is called "sewage fungus." It is also responsible for bulking in poorly aerated or overloaded aerobic tanks. Some microaerophilic bacteria participate in the transformation of metals. Microaerophilic spirilla, for example *Magnetospirillum spp.*, can produce magnetite from Fe<sup>2+</sup>.

Microaerophiles can form H<sub>2</sub>O<sub>2</sub> and other reactive oxygen species (ROS), such as superoxide radical and hydroxyl radical as the final products of oxygen detoxication. ROS can oxidize nonspecific xenobiotics. Microaerophilic bacteria are known to cause the biodegradation of benzene, phenol, toluene and naphthalene.

Together with the species useful for environmental biotechnology, there are many pathogenic microaerophilic organisms, for example, some strains of *Escherichia coli* that cause intestinal infections, *Salmonella spp.* and *Campylobacter spp.*, that cause life-threatening infections salmonellosis and campylobacteriosis, respectively. Some strains of bacteria from the species *Vibrio cholera* are agents of water-borne infectious disease. Bacteria *Helicobacter pylori* cause stomach ulcer, and *Treponema pallidum* cause syphilis. Some facultative anaerobic bacteria, for example, *Stenotrophomonas maltophilia*, are active degraders of xenobiotics but are opportunistic pathogens. These strains are used in environmental engineering but strict biosafety rules must be heeded in their applications.

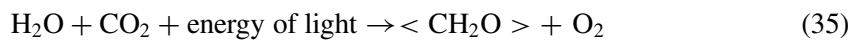
*Chemotrophic subline of Firmicutes (group P3-L2c).* Microaerophilic filamentous Gram-positive bacteria, for example, *Microthrix parvicella*, *Nocardia spp.*, *Trichococcus*, are common to wastewater-activated sludge; however, the abundance of these organisms is associated with bulking, foaming and scum formation, and finally with wastewater treatment failure.

There are facultative anaerobic Gram-positive bacteria belonging to the genera *Propionibacterium*, *Staphylococcus*, *Streptococcus* and *Enterococcus*, which are closely associated with the surfaces of human body and animals. Some are the agents of infectious diseases. These bacteria, for example, *Enterococcus*, are used in environmental engineering as indicators of bacteriological quality of the environment.

Facultative anaerobic species among *Actinomycetes*, a group of Gram-positive bacteria with high G + C content in DNA, are important in the degradation of organic compounds in soil. The microaerophilic representatives of genus *Frankia* are able to fix molecular nitrogen in symbiosis with nonleguminous plants.

*Chemotrophic subline of Archaea (group P3-L3c)*. The species from genera *Sulfolobus*, *Acidianus* and *Metallosphaera* are microaerophilic or facultative anaerobic, grow in sulphur-rich, hot acid biotops and are capable of oxidizing organic substances, S,  $S_4O_6^{2-}$ ,  $S^{2-}$  and  $Fe^{2+}$ , using oxygen or  $Fe^{3+}$  as electron acceptors. Another group of facultatively anaerobic, thermoacidophilic *Archaea*, are the species of the genera *Thermoplasma*, *Picrophilus* and *Ferroplasma*. Applications of acidophilic *Archaea* in environmental engineering may be bioextraction (bioleaching) of heavy metals from sewage sludge at high temperatures. Another potential application is the bioremoval of inorganic and organic sulphur from coal and oil to diminish the emissions of sulphur oxides in the atmosphere.

*Phototrophic subline of Gracilicutes (group P3-L1p)*. The oxygenic photosynthetic Gram-negative bacteria comprise the cyanobacteria and the prochlorophytes that are distinguished by their photosynthetic pigments. Some representatives from the *Microcoleus* and *Oscillatoria* genera are facultative anaerobic organisms capable of anoxygenic photosynthesis with hydrogen or sulphide as electron donors for the reduction of  $CO_2$  or for oxygenic photosynthesis:



where  $<CH_2O>$  is the conventional formula for synthesized carbohydrates.

The ability of cyanobacteria to grow in both aerobic and anaerobic environments is related to the life of some cyanobacteria in a dense microbial mat, where the conditions are aerobic during the day and become anaerobic at night. During anoxygenic photosynthesis under  $CO_2$  limitation, the electrons from sulphide may be also used for fixation of molecular nitrogen or for the production of molecular hydrogen. This feature can be used in the biogeneration of hydrogen in fuel cells.

*Phototrophic subline of Firmicutes (group P3-L2c)*. There are no currently known microaerophilic or facultatively aerobic, Gram-positive phototrophic bacteria.

#### 5.4. Functions of Aerobic Prokaryotes

*Chemotrophic subline of Gracilicutes (group P4-L1c)*. Strictly speaking, aerobic chemotrophic Gram-negative bacteria are the most active organisms in the biodegradation of xenobiotics, aerobic wastewater treatment, and soil bioremediation. Examples of the functions of selected genera are shown below.

Members of the genus *Pseudomonas* (the pseudomonads), for example *P. putida*, *P. fluorescens* and *P. aeruginosa*, are used in environmental biotechnology as active degraders of xenobiotics in wastewater treatment and soil bioremediation. These organisms oxidize aliphatic

hydrocarbons, monocyclic and polycyclic aromatic hydrocarbons, halogenated aliphatic and aromatic compounds, different pesticides and oxidize or cometabolize halogenated ethanes and methanes. Biodegradation often depends on the presence of specific plasmids. Therefore, both native and genetically engineered strains with amplified and diverse degradation ability are used in environmental engineering. Some selected strains of *Pseudomonas* genus are used instead of chemical biocides to control plant diseases. Other active biodegraders are the species from genera of *Alcaligenes*, *Acinetobacter*, *Burkholderia*, *Comamonas* and *Flavobacterium*. The majority of active biodegraders are opportunistic bacteria, i.e., they can cause diseases in immunosuppressive, young or old people. Therefore, all experiments and treatments of water and soil by these bacteria must be performed with precautions against the dispersion of these bacteria in the environment and reasonable biosafety rules must be used in their applications.

There are many pathogens among the above-mentioned genera, for example, *Pseudomonas aeruginosa*, *Burkholderia cepacia*, and *Burkholderia pseudomallei*. Therefore, the test of acute toxicity and other pathogenicity tests of all microbial strains, isolated as the active biodegraders of xenobiotics for environmental engineering applications, must be made after selection and before pilot-scale research. Representatives of genus *Xantomonas* are also active biodegraders, but there are many phytopathogenic species. Therefore, they cannot be used for soil bioremediation, but used as the test cultures to test new biocides for agriculture.

The formation of activated sludge flocs is enhanced by the production of extracellular slime. *Zoogloea ramigera* is considered an important organism in flocs formation because of its strong self-aggregation. Probably the most important role in the formation of activated sludge floc belongs to gliding bacteria from the genera *Flavobacterium*, *Cytophaga*, *Myxobacterium*, *Flexibacterium* and *Comamonas*. They are called gliding bacteria because of cell translocation on a solid surface due to the interaction of cell surface and solid surface. They produce extracellular polysaccharides and have the ability for strong aggregation of their cells.

Species of genus *Acinetobacter* are capable of accumulating polyphosphate granules and are used for biological removal of phosphate from wastewater. This removal diminishes the supply of phosphate ions and dissolved phosphates of heavy metals to the environment. Another important environmental biotechnology feature of these bacteria is the ability to produce extracellular polyanionic heteropolysaccharides that can emulsify hydrocarbons, and thus, enhance their degradation in an aqueous environment. Together with these useful properties, *Acinetobacter spp.* are opportunistic pathogens that cause different infections in immunocompromised patients. These infections are difficult to treat successfully because clinical strains of *Acinetobacter ssp.* acquire resistance to the antibiotics.

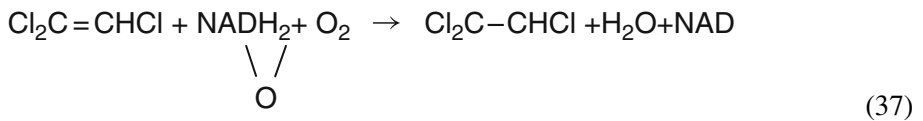
*Azotobacter* and *Azomonas* are the genera of free-living nitrogen fixing soil bacteria that accumulate organic nitrogen and improve soil fertility. Selected strains of genus *Azotobacter* are used for the industrial biosynthesis of PHB and its derivatives for biodegradable plastics. Biodegradability of plastic materials is very important for environmental sustainability.

Methanotrophs do not grow on organic compounds and oxidize such single-carbon compounds as methane, methanol, formaldehyde and formate. Methane-oxidizing species, for example, from the *Methylococcus* and *Methylomonas* genera, are important for the removal of methane from the atmosphere, thus, diminish the greenhouse effect due to accumulation of

carbon dioxide and methane:



A well known application in environmental biotechnology is the bioremoval of halogenated methanes and ethanes from the polluted groundwater by cometabolism. For example, cometabolism of trichloroethylene (TCE) by methylotrophic bacteria is considered an effective approach for the remediation of a polluted aquifer. The main reaction of cometabolism, catalyzed by enzyme methanemmonooxygenase of methanotrophs, is described by the following equation:



There are many oligotrophes in the group P4-L1. These organisms are adapted to living in a poor environment with a low concentration of nutrient sources, including carbon and energy. Their adaptation is so stable that many oligotrophes are obligate ones and cannot grow in a medium with a high concentration of carbon and energy sources. Oligotrophic microorganisms are important for the treatment of ground water, sea water and fresh water with a low concentration of carbon source. For example, *Hyphomicrobium spp.* are budding oligotrophic bacteria capable to oxidize single-carbon compounds by oxygen or nitrate. They are used in environmental biotechnology for the removal of nitrate from water, using methanol as an electron donor. Stalked oligotrophic from genus *Caulobacter spp.* are able to survive during long-term starvation. It is thought that they may perform gene transfer between different bacteria participating in water and wastewater treatment because they are often adhered to the cells of other bacteria.

*Bdellovibrio spp.* are Gram-negative bacteria, which are parasites of other Gram-negative bacteria. Therefore, their presence in water is an indication of water pollution by Gram-negative bacteria. *Bdellovibrio spp.* can be applied in environmental engineering for the biological control of human, animal and plant pathogens in water and soil, and for control of excessive growth of microbial biofilm in fixed biofilm reactors, which are used for water and wastewater treatment.

Rhizobia are bacteria from the genera *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, which are able to grow and fix atmospheric molecular nitrogen symbiotically with leguminous plants, for example, peas, beans and clover. It is considered that this fixation supplies about a half the nitrogen used in agriculture. The application of specifically selected and industrially cultivated rhizobia for the inoculation of soil, where leguminous plants are planted for the first time, can double the yield of these plants. Therefore, these bacteria are used in environmental engineering for the enhancement of soil fertility after bioremediation of polluted soil.

There are many pathogenic species in the group P4-L1. *Yersinia pestis* is responsible for the devastating outbreaks of plague in Asia and Europe in the sixth, fourteenth, seventeenth, eighteenth and beginning of the twentieth centuries. Another example is *Burkholderia pseudomallei*, agent of melioidosis. This disease is common in Southeast Asia. It affects people exposed to soil and soil aerosols: farmers on rice paddies, construction workers or people

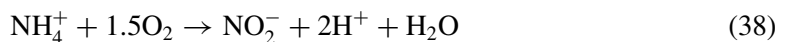
living close to the soil excavation area. The disease may be misidentified as syphilis, typhoid fever or tuberculosis. Pulmonary melioidosis can range from bronchitis to a severe pneumonia. During the period from 1989 to 1996, a total of 372 melioidosis cases, with 147 deaths, were reported in Singapore (15). *Legionella spp.* is an agent of Legionnaires disease, which is a lung infection caused by inhalation of water droplets from poorly maintained cooling towers, air conditioners, fountains and artificial waterfalls.

Pathogens can be removed or killed in environmental engineering systems by the following methods:

1. Bulk or membrane filtration, and UV treatment for air and aerosol disinfection.
2. Coagulation, aggregation, sedimentation, slow filtration of water and wastewater to diminish the concentration of pathogens due to adsorption, sedimentation of microbial aggregates or predation of protozoa.
3. Chemical treatment by chlorine, chlorine dioxide, ozone or UV light for water and wastewater effluent disinfection.
4. Chemical treatment by oxidants, organic solvents, surfactants, salts of heavy metals or UV light for the disinfection of solid surfaces and microbial biofilms.
5. Aeration, biotreatment, thermal treatment, acidification, hydrogen peroxide addition, electromagnetic radiation to disinfect or to diminish the content of pathogens in solid waste or soil.

*Chemolithotrophs in chemotrophic subline of Gracilicutes (subgroup of the group P4-L1c).* Chemolithotrophs are aerobic prokaryotes, which can use a reduced inorganic compound as an energy source. The species grow as autotrophs but some can also grow as organotrophs.

Nitrifying bacteria comprise two groups of aerobic bacteria: ammonia-oxidizers (*Nitrosomonas spp.*, *Nitrosococcus spp.*, *Nitrosovibrio spp.*, *Nitrosospira spp.*, *Nitrosolobus spp.*) performing the reaction:



and nitrite-oxidizers (*Nitrobacter spp.*, *Nitrococcus spp.* and *Nitrospira spp.* from different subdivisions of 16S rRNA classification) that use the nitrite to form nitrate:



Nitrifying bacteria are widely used in environmental biotechnology to transform toxic ammonium to a less toxic nitrate. The nitrate produced can be transformed further to molecular nitrogen by denitrifying bacteria. The problems of large-scale nitrification in wastewater treatment include:

1. Washing out of these microorganisms from a bioreactor of continuous cultivation due to their slow growth rate in comparison with the growth rate of heterotrophs.
2. High sensitivity of nitrifiers to toxic substances, surfactants or organic solvents due to the large folded surface of a cell membrane.

The activity of nitrifying bacteria was the basis of ancient Chinese environmental biotechnology to convert organic wastes and household ashes into fertilizer and gunpowder as a value-added by-product. The transformation of waste to gunpowder can be described by the following sequence of the reactions:



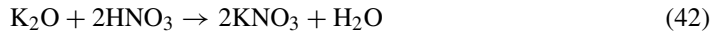
1. Formation of ammonia from aminoacids of proteins by bacteria-ammonifiers in anaerobic microzones of waste



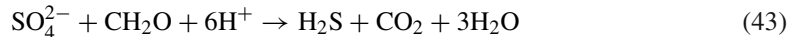
2. Production of nitrate from ammonium by bacteria-nitrifiers in aerobic microzones of waste



3. Neutralization of acid solution of nitrate by potassium oxide from ash



4. Formation of hydrogen sulphide by sulphate-reducing bacteria in anaerobic microzones of waste



5. Oxidation of hydrogen sulphide by microaerophilic bacteria



These reactions result in a suspension, containing carbon particles from ash, particles elemental sulphur and potassium nitrate. Drainage of this suspension from the waste pile and drying it under the sun in a drainage collector, produce a mixture of carbon, sulphur and potassium nitrate, which is gunpowder.

Aerobic sulphur-oxidizing chemolithotrophic bacteria oxidize reduced sulphur compounds, producing sulphuric acid:



These bacteria are used or can be used in environmental biotechnology for the following purposes:

1. Bioremoval of toxic  $\text{H}_2\text{S}$  from gas, water and wastewater.
2. Industrial bioleaching of metals, for example, copper, zinc, or uranium from the ores.
3. Bioleaching of heavy metals from anaerobic sewage sludge before its utilization as an organic fertilizer.
4. Bioleaching of heavy metals and radionuclides from polluted soil.
5. Acidification of alkaline soil.

Sulphur oxidation can cause corrosion of steel and concrete constructions due to the production of sulphuric acid. Some species, for example, *Thiobacillus ferrooxidans*, (*Acidithiobacillus ferrooxidans*), can grow at an extremely low pH and be isolated from acid mine drainage.

Oxidization of Fe(II) is performed by two groups of aerobic bacteria: acidophilic and neutrophilic iron-oxidizers. Fe(II) is stable in acid solutions, if the pH is lower than 3. Its chemical oxidation by oxygen of air under a low pH is slow. Some bacteria, however, for example, *Thiobacillus ferrooxidans*, (*Acidithiobacillus ferrooxidans*), can oxidize Fe(II) some thousand times faster than that in a chemical process. Neutrophilic iron- and manganese-oxidizers are usually microaerophilic, and were described above as the representatives of

group P3-L1. The main point for neutrophilic oxidation of iron is that Fe(II) is not stable under aerobic conditions and neutral pH and must be immediately oxidized by oxygen. However, atoms of Fe(II), surrounded by chelated organic acids, are protected from chemical oxidation by oxygen. Therefore, the functions of neutrophilic microaerophilic “iron-oxidizers” are production of H<sub>2</sub>O<sub>2</sub> and chemical degradation of organic “envelope” of Fe<sup>2+</sup> atom by H<sub>2</sub>O<sub>2</sub>. Precipitation of iron hydroxide by these bacteria can clog pipelines and wells. They are used in environmental engineering for the removal of iron and manganese from drinking water, treated in slow sand filter. Another important application is the removal of ammonia from wastewater by coprecipitation with fine particles of positively charged iron hydroxide produced by neutrophilic “iron-oxidizers” (12).

*Chemotrophic subline of Firmicutes (group P4-L2c).* Aerobic heterotrophic Gram-positive bacteria have diverse functions in environmental biotechnology. Representatives of genus *Bacillus* dominate in the aerobic treatment of wastewater or solid waste, which is rich in such polymers as starch or protein. The species of genus *Bacillus* produce antibiotics and can degrade different xenobiotics. They produce endospores providing cell survival in variable soil environment and after drying. Therefore, the endospores can be used in commercial compositions to start up soil bioremediation or biodegradation of certain substances during wastewater treatment. The shape and cellular location of endospores are used as identifying characteristics in the differentiation of the species. Some species, for example *Bacillus subtilis*, live in human intestine and are used as a medical application of viable cells as probiotics to normalize microflora of intestine. However, there are also pathogenic species in this genus. For example, *Bacillus anthracis* cause deadly infection anthrax due to the production of strong toxins and are even considered as biological weapons.

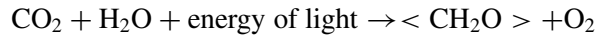
Bacteria of the genus *Arthrobacter* are commonly found in soil and are active in the biodegradation of xenobiotics. Their specificity is a rod-coccus growth cycle; the cells are rod-shaped during active growth and cocci-shaped in the stationary phase of batch culture. Saprophytic species of mycobacteria are skilled degraders of xenobiotics but there are also many pathogenic species, for example, *Mycobacterium tuberculosis* (agent of tuberculosis) and *Mycobacterium leprae* (agent of lepra). *Nocardia spp.* and *Rhodococcus spp.* degrade hydrocarbons and waxes; they form mycelium, which breaks into rods and cocci. Excessive growth of *Nocardia asteroides*, *Rhodococcus spp.*, and *Gordona amarae* in aerobic tank causes foaming (brown scum) of activated sludge due to high hydrophobicity of cell surface of these species, their production of biosurfactants, or hydrolysis of lipids. This foaming produces nuisance odours and can increase the risk of infection of wastewater workers with opportunistic pathogenic actinomycetes *Nocardia asteroides*.

*Actinomycetes* are aerobic, heterotrophic Gram-positive prokaryotes with a high G + C content in DNA, growing with aerial mycelia. They are used in the aerobic treatment of wastewater, soil bioremediation, and composting of solid wastes because there are many active degraders of natural biopolymers and xenobiotics. Genus *Streptomyces*, containing half a thousand species, is extremely important for medical biotechnology because many antibiotics are produced by the strains from this genus. A significant part of soil microbial biomass is the biomass of streptomycetes. Therefore, the character odour of moist earth is due to the volatile

substances such as geosmin produced by these microorganisms. Thermophilic actinomycetes play an important role in the composting of organic wastes.

*Chemotrophic subline of Archaea (group P4-L3c)*. Some halophilic Archaea are aerobic microorganisms. Hypothetically, they can be used for the biotreatment of polluted industrial brines but there are no applications of these prokaryotes in environmental biotechnology at present.

*Phototrophic subline of Gracilicutes (group P4-L1p)*. Cyanobacteria carry out oxygenic photosynthesis with water as the electron donor:



where  $\langle \text{CH}_2\text{O} \rangle$  is the conventional formula of photosynthesized carbohydrates. Cyanobacteria are used in environmental biotechnology for the light-dependant removal of nitrogen and phosphorus from wastewater. However, a main problem of environmental engineering, related to cyanobacteria, is control of their blooming in surface water polluted with ammonia or phosphate. This bloom causes nuisance odours, bad taste of water, and accumulation of allergens and toxins in water. The cyanobacteria are morphologically diverse including unicellular organisms reproduced by binary fission or budding (*Chroococcales*), by multiple fission (*Pleurocapsales*), and filamentous forms without cell differentiation (*Oscillatoriales*) or with cell differentiation (*Nostocales* and *Stigonematales*). In the 16S rRNA classification of cyanobacteria, the cyanobacteria are clustered in 14 sections (16).

Prochlorophytes is a group of aerobic oxygenic phototrophic Gram-negative bacteria that differ from cyanobacteria by their set of photosynthetic pigments. Group *Prochloron* is close to cyanobacteria by 16S rRNA phylogeny. This group has no importance for environmental engineering because they have been found only as extracellular symbionts of ascidians, marine animals, in the tropical areas of Pacific and Indian oceans.

*Phototrophic subline of Firmicutes (group P4-L2p)*. There are no currently known obligate aerobic Gram-positive phototrophic bacteria.

### 5.5. Functions of Eukaryotic Microorganisms

*Fungi* are used in environmental biotechnology in the composting, soil bioremediation and biodegradation of xenobiotics in the soil. Activated sludge of municipal wastewater treatment has a low content of fungi from the genera *Geotrichum*, *Penicillium*, *Cephalosporium*, *Cladosporium* and *Alternaria*, but the matrix of such microbial aggregates as granules and biofilms can be arranged due to the mycelium of fungi from genera *Fusarium*, *Penicillium*, *Aspergillus*, *Mucor* and *Geotrichum*. The mycelial structure of biofilm facilitates transfer of oxygen and nutrients to the deeper layers of the biofilm. Fungi dominate in the microbial ecosystems with a low pH. Therefore, there may be sludge bulking at a low pH due to the excessive growth of fungi. The hydrolyzing activity of fungi is essential for composting of such organic wastes as municipal refuse, paper, sewage sludge, agricultural and farming wastes, and food-processing waste. The objective of composting is to convert an unstable and unsafe organic waste into a dark-brown, granular, humus-like end-product, with a high content of nutrients, which can be applied as a soil conditioner. Heat produced during composition destroys human pathogens and parasites. Without proper control of composting, the

production of odours of microbial origin (volatile fatty acids, hydrogen sulphide), air-spread spores of fungi, for example, opportunistic pathogen *Aspergillus fumigatus* or *Aspergillus flavus*, producer of cancerogenic aflatoxin, may create safety problems. There are many other species of fungi growing on the surfaces and producing mycotoxins released to aquatic systems and air-spread spores that cause respiratory infection and allergic reactions.

*Algae* grow on biofilm surfaces exposed to light and used in water or wastewater treatment. They consume nitrogen and phosphate and produce oxygen used by aerobic bacteria and fungi. However, environmental engineers are interested mostly in the control of algae in aquatic systems because some species produce toxins and promote the growth of pathogens in water.

*Protozoa* are unicellular animals that obtain nutrients by ingesting food particles or microorganisms. Some protozoa can form cysts under adverse environmental conditions. These cysts are resistant to desiccation, starvation, high temperature and disinfection. Changes in the protozoan community reflect the operating conditions of aeration tank and are used for microscopic monitoring of the process in aerobic tanks. Amoeba dominate under a high concentration of organic matter, and ciliates, while flagellates, dominate under lower concentrations. The presence of stalked ciliates attached to the surface of the flocs is a sign of low concentration of organic substances in treated wastewater. Ciliates feed on suspended bacterial cells, thus improving effluent quality. Some functions have rotifers, which are multicellular organisms with size from 50 to 250  $\mu\text{m}$ , attached to the flocs of activated sludge. Predation by protozoa and rotifers can diminish the concentration of pathogens in water during wastewater treatment. From another view, some protozoa are natural hosts of bacterial pathogens, for example, *Legionella spp.* The pathogenic strains of these bacteria grow and survive in cells of protozoa living in warm water. Therefore, the growth of these protozoa must be controlled using biocides or high temperature to prevent outbreaks of Legionnaires disease.

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